

# **Retinoid and Pentoxifylline-Induced Modulation of Human Melanoma Cell Metastasis**

by

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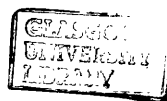
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Dedicated to my mother and father  
Margaret and Denis Alexander

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## **Abbreviations**

AF	activation function
AMF	autocrine motility factor
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5' cyclic monophosphate
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CTL	cytotoxic T lymphocyte
DGEA	aspartate-glycine-glutamate-alanine
dNTP	2' deoxyribonucleotide triphosphate
DR	direct repeat
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E:T	effector to tumour
FAK	focal adhesion kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycan
Gly	glycine
GPI	glycosyl phosphatidyl inositol
HGF	hepatocyte growth factor
hsp	heat shock protein
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LAK	lymphokine activated killer
LDL	low density lipoprotein
LFA	lymphocyte function associated antigen
LRP	low density lipoprotein-related protein

MEM	minimal essential medium
MHC	major histocompatibility complex
mICAM-1	membrane intercellular adhesion molecule-1
MOPS	3' [N-morpholino]propanesulphonic acid
MMP	matrix metalloproteinase
NCAM	neural cell adhesion molecule
NK	natural killer
NF	nuclear factor
PAI	plasminogen activator inhibitor
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDSGR	proline-aspartate-serine-glycine-arginine
PKC	protein kinase C
PMA	phorbol myristate acetate
PVP	polyvinylpyrrolidone
PX	pentoxifylline
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
R-FA	fatty acid retinyl esters
RGD	arginine-glycine-asparagine
RXR	retinoid X receptors
RYVVLPR	arginine-tyrosine-valine-valine-leucine-proline-arginine
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
sICAM-1	soluble intercellular adhesion molecule-1
SSC	saline-sodium citrate
SSPE	saline-sodium phosphate-EDTA
STAT	signal transducer and activator of transcription

TBE	tris-borate-EDTA
TcR	T cell receptor
TGF	tumour derived growth factor
tPA	tissue type plasminogen activator
TR	thyroid hormone receptor
uPA	urokinase type plasminogen activator
uPAR	urokinase plasminogen activator receptor
VCAM-1	vascular cell adhesion molecule-1
VDR	vitamin D receptor
YIGSR	tyrosine-isoleucine-glycine-serine-arginine

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## **Summary**

Successful metastasis of a tumour cell involves a series of complex steps comprising of several cell-cell and cell extracellular matrix (ECM) interactions. All-trans retinoic acid (RA) and pentoxifylline (PX) have previously been shown to modulate various stages of metastasis, and this study examines the ability of either drug to modulate tumour cell growth, adhesion of tumour cells to various components of the ECM, tumour lysis by lymphokine-activated killer (LAK) cells, and plasminogen activator expression. Pretreatment of the A375, Hs294T and C8161 human melanoma cell lines for up to 7 days with a range of concentrations of RA ( $10^{-10}\text{M}$  -  $10^{-5}\text{M}$ ) failed to alter tumour cell growth. In contrast, PX induced a time and dose dependent decrease in tumour cell numbers, reducing C8161 cells numbers by 67.5%, Hs294T cell numbers by 65.4% and decreasing the numbers of A375 cells by 37.2% after 4 days treatment with  $250\mu\text{g/ml}$  PX. Neither PX nor RA had any effect on tumour cell morphology. Pretreatment of the C8161 cells for 4 days with  $10^{-6}\text{M}$  RA had no effect on adhesion of the cells to fibronectin, laminin, collagens type I and type IV or matrigel basement membrane. Although adhesion of C8161 cells pretreated with  $250\mu\text{g/ml}$  PX to collagens type I and IV, and laminin was not altered, a 24.2% reduction in adhesion to fibronectin was observed by 30min.

The ability of either drug to modulate tumour cell lysis by LAK cells was determined using the Chromium-51 release assay over a range of effector to tumour cell ratios (E:T). Neither drug modulated lysis of the A375 cells but lysis of the Hs294T and C8161 cells pretreated for 4 days with  $10^{-6}\text{M}$  RA was increased 1.4-fold and 2.8-fold respectively at an E:T ratio of 40:1. 4 days pretreatment of the tumour cells with PX ( $250\mu\text{g/ml}$ ) failed to alter lysis of the A375 cells yet increased lysis of the C8161 cells 1.8-fold and in contrast, decreased lysis of the Hs294T cells 1.8-fold at the 40:1 ratio. The involvement of intercellular adhesion molecule-1 (ICAM-1, CD54) in the modulation of tumour cell lysis by either RA or PX was subsequently examined. ICAM-1 is expressed on several cell types including melanoma cells and acts as a ligand for lymphocyte function associated antigen (LFA-1, CD11a/CD18) present on the surface of lymphocytes. Analysis of alterations in membrane ICAM-1 (mICAM-1) by flow cytometry revealed RA ( $10^{-10}\text{M}$  -  $10^{-7}\text{M}$ ) had no effect on mICAM-1 expression in the Hs294T and C8161 cells whereas at the higher concentrations ( $10^{-6}\text{M}$  -  $10^{-5}\text{M}$ ), a slight increase in mICAM-1 levels became evident. Using the A375 cell line, 4 days pretreatment with  $10^{-10}\text{M}$  -  $10^{-7}\text{M}$  RA induced

only a marginal increase in mICAM-1 expression, which further increased with  $10^{-6}\text{M}$  -  $10^{-5}\text{M}$  RA. Despite the RA-induced increase in mICAM-1 levels, PX ( $10\mu\text{g/ml}$  -  $250\mu\text{g/ml}$ ) failed to alter mICAM-1 expression in any of the three cell lines. Addition of blocking ICAM-1 antibody over a range of concentrations to the cytotoxicity assays at the 40:1 ratio reduced lysis of the C8161, A375 and Hs294T cells. Using the C8161 cells, blocking ICAM-1 antibody at the optimum concentration of  $10\mu\text{g/ml}$  induced a 2.3-fold reduction in lysis of the tumour cells. In the Hs294T cell line, an optimum 1.8-fold reduction in lysis was observed using  $5\mu\text{g/ml}$  blocking ICAM-1 antibody. Using the A375 cells,  $10\mu\text{g/ml}$  blocking ICAM-1 antibody induced an optimum 2-fold reduction in lysis. This antibody also reduced lysis of RA-pretreated C8161 cells 3-fold using  $10\mu\text{g/ml}$  antibody, and the Hs294T cells 1.3-fold using  $5\mu\text{g/ml}$ . Lysis of PX-pretreated C8161 and Hs294T cells was also reduced 2.1-fold ( $5\mu\text{g/ml}$ ) and 1.5-fold ( $10\mu\text{g/ml}$ ) respectively. Despite showing weak expression of the major histocompatibility complex (MHC) class 1 on all 3 cell lines, blocking of this molecule using a specific antibody had no effect on tumour cell lysis. Collectively, this data shows that ICAM-1 is partially involved in the lysis of RA/PX-treated and untreated tumour cells but other molecules involved in the tumour/LAK cell interaction must also be altered to account for the observed RA/PX induced modulation of tumour lysis.

ICAM-1 can also exist as a soluble form (sICAM-1) which has the ability to bind to LFA-1 thereby blocking the binding site of LFA-1 for membrane ICAM-1 on the tumour cell surface. This prevents the tumour cells from being lysed by the lymphocytes and is thought to be one possible mechanism as to how tumour cells evade immunosurveillance. Using a specific ELISA detection system, expression of sICAM-1 was measured from each cell line. The A375 cells secreted  $50.5\text{fg/cell}$ , compared to  $36.9\text{fg/cell}$  for the C8161 cells, and  $7.2\text{fg/cell}$  for the Hs294T cells. RA ( $10^{-10}\text{M}$  -  $10^{-5}\text{M}$ ) did not alter sICAM-1 expression in the C8161 and Hs294T cells, but in contrast, levels were increased in the A375 cells 1.4-fold using  $10^{-6}\text{M}$  RA. PX ( $10\mu\text{g/ml}$  -  $250\mu\text{g/ml}$ ) induced a dose-dependent increase in sICAM-1 expression in all three cell lines. Using  $250\mu\text{g/ml}$  PX, sICAM-1 expression was increased 4.3-fold, 4.7-fold, and 1.7-fold in the C8161, Hs294T and A375 cells respectively. Addition of sICAM-1 to the cytotoxicity assays of C8161 and Hs294T cells had no effect on tumour cell lysis. In contrast, addition of the soluble form to RA-pretreated C8161 cells induced a 3.1-fold reduction in lysis yet did not alter lysis of the Hs294T cells. This shows that sICAM-1 appears to be unimportant in the evasion of the C8161 and Hs294T

cells, but in combination with alterations in cell surface molecules, may play a role in escape of RA-pretreated C8161 cells from the immune system.

As well as the heterotypic cell interactions occurring during the metastatic process, tumour cells must also have the ability to interact with and invade the surrounding tissues. The plasminogen activation system consists of a series of secreted enzymes which play a pivotal role in invasion by the tumour cell. Two well documented plasminogen activators are urokinase type (uPA) and tissue type (tPA) plasminogen activators which convert plasminogen into the active zymogen plasmin which has a broad substrate specificity degrading a wide variety of ECM components. Using confocal microscopy, it was shown that all 3 cell lines expressed cell-associated uPA although expression was much stronger in the Hs294T and C8161 cells compared to the A375 cells. tPA was also found to be associated with greater than 95% of the Hs294T, C8161 and A375 cells, with the staining being much weaker in the Hs294T and C8161 cells than the uPA staining. A specific ELISA was used to detect secreted uPA and tPA expression from each cell line. Levels of secreted uPA were undetectable in the A375 cell line, whereas, in contrast, the highly metastatic C8161 cell line was found to secrete on average 215-fold higher levels of uPA than the poorly metastatic Hs294T cell line. Expression of secreted tPA was 6.4-fold and 6.9-fold higher in the A375 and Hs294T cells respectively than in the C8161 cells. RA ( $10^{-10}\text{M}$  -  $10^{-5}\text{M}$ ) failed to induce expression of uPA from the A375 cells. Nevertheless, the same treatment of the C8161 cells induced a maximum 3.4-fold increase in uPA expression using  $10^{-8}\text{M}$  RA compared to a 1.6-fold increase in the Hs294T cells. Secretion of tPA was increased with  $10^{-8}\text{M}$  -  $10^{-5}\text{M}$  RA pretreatment of the C8161, achieving a maximum 3.7-fold increase using  $10^{-6}\text{M}$  RA. In the Hs294T cell line, tPA secretion was increased with  $10^{-9}\text{M}$  -  $10^{-5}\text{M}$  RA where an optimum 3.8-fold increase in tPA expression was induced using  $10^{-6}\text{M}$  RA. Preliminary measurements of the modulation of uPA and tPA expression by PX ( $10\mu\text{g/ml}$  -  $250\mu\text{g/ml}$ ) indicated PX treatment of the C8161 and Hs294T cells induced an increase in uPA expression 2.6-fold and 2.7-fold respectively. The effect of PX on secreted tPA expression in the A375 and Hs294T cells has still to be investigated. In the C8161 cells, the PX-induced change in tPA expression was marginal, where expression decreased 1.4-fold using  $250\mu\text{g/ml}$  PX. The biological significance of these alterations in uPA and tPA expression by RA and PX remains to be examined. The retinoid-induced changes in tumour LAK cell-mediated lysis and plasminogen activation and the apparent lack of alterations in adhesive interactions in the cell lines led

to an interest in the mechanisms involved in these observed responses. All-trans RA exerts its effect via 3 subtypes of nuclear receptor namely RAR $\alpha$ ,  $\beta$  and  $\gamma$  which can form homo or heterodimeric complexes with the retinoid X receptors (RXR). The receptors exert their physiological actions by binding to specific sites on the DNA termed the retinoic acid response element (RARE) on target genes to regulate their expression. Using the polymerase chain reaction (PCR) to amplify specific regions of the cDNA from each cell line, followed by Southern blot analysis to confirm the identity of the amplicons, it was shown that all three cell lines expressed RAR $\beta$  and  $\gamma$  with and without 4 days pretreatment with 10<sup>-6</sup>M RA. In contrast the C8161 and Hs294T cells lacked expression of RAR $\alpha$  with and without RA pretreatment whereas, RAR $\alpha$  was expressed in the A375 cells but was down regulated on treatment with 10<sup>-6</sup>M RA. In relation to the studies on ICAM-1 and PA, RAR $\beta$  has been shown to be involved in the upregulation of mICAM-1 by RA, and certainly all 3 cell lines expressed this subtype allowing for the potential of RAR $\beta$  to be involved in the RA-induced increase in mICAM-1 levels in all 3 cell lines. The RA induction of tPA expression in other cell types has been reported to involve RAR $\alpha$ , and as all 3 cell lines lacked expression of this subtype on treatment with RA, this suggests other mechanisms must exist, possibly involving RAR $\beta$  and  $\gamma$  to regulate tPA expression by RA.

## **Chapter 1: Introduction**

## **1. General Introduction**

### **1.1 Development of a malignant tumour**

The development of a malignant tumour is a three step process involving initiation, promotion and progression (Weinstein IB, 1988). The transformation events can be initiated by genetic predisposition, exogenous factors such as chemical carcinogens, and endogenous factors including hormones. The growth and expansion of the initiated cell occurs during the promotion phase which is followed by progression leading to a genetically unstable cell. During this progression, the cells undergo several characteristic and phenotypic changes involving deletions in putative suppressor gene loci (Shih and Herlyn, 1993), the acquired loss of dependence of exogenous growth factors, and loss of susceptibility to negative growth regulators (Rodeck U, 1993; Lu and Kerbel, 1994). Once the transformation events have taken place, the resulting neoplastic cells proliferate to form the primary tumour. The genetic instability of the cells within a tumour contributes to the generation of heterogeneity which may determine the ability of a tumour cell to metastasise.

### **1.2 Steps involved in metastasis**

The term metastasis, which describes the spread of malignant cells to secondary sites throughout the body, was first coined in 1829 by the French physician Joseph Claude Recamier. He made the observation that patients with breast cancer also had secondary deposits on the brain and noted many of the veins located near the affected sites were invaded with tumour cells. Most deaths in cancer are due to metastatic disease which involves a series of sequential steps (Liotta and Stetler-Stevenson, 1991), beginning with the dissociation of the tumour cells from the main tumour mass. This is followed by adhesion to and penetration of basement membrane barriers during vascular and lymphatic invasion. Tumour cells are carried in the circulation where they must escape immunosurveillance and then penetrate blood vessel walls of target organs and invade the surrounding tissue. Once they finally establish at a distant site, the tumour cells begin to proliferate and induce their own blood supply, a process called angiogenesis.

### *Adhesion Events*

The importance of cell adhesion to the process of metastasis is well documented (Pignatelli and Vessey, 1994). Reduced cell adhesion must occur to allow tumour cell detachment from the primary mass yet there needs to be an increase in cell adhesive properties to promote attachment of tumour cells to microvessel endothelial cells, subendothelial basement membrane or the extracellular matrix (ECM) during invasion. A decrease in adhesion is aided by reduction or loss of specific cell adhesion molecules such as the cadherins, where loss of expression of E-cadherin has been correlated with poor prognosis of high grade breast cancer tumours (Oka et al, 1993). Tumour cell adherence to endothelial cells is mediated by the immunoglobulin superfamily of adhesion molecules which are involved primarily in cell-cell communication. 2 members of this family, ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) have been implicated in the progression of neoplasms, particularly melanoma (Johnson et al, 1989; Denton et al, 1992). In contrast, interaction of the tumour cells with the ECM is mediated largely by integrins which allow the tumour cells to interact with a variety of components of the ECM including fibronectin, laminin, collagens, proteoglycans and vitronectin. Changes occurring in adhesion receptors as a consequence of transformation tend to be tumour type and integrin specific (Juliano and Varner, 1993). Tumour cell adhesion to the ECM may also provide a signal for secretion of autocrine and /or paracrine growth factors resulting in enhanced tumour cell proliferation (Nicolson GL, 1993) and can trigger a host of other cellular functions including cell migration, release of ECM-degrading enzymes, and the release of matrix-bound angiogenic factors such as basic fibroblast growth factor (bFGF) which are required for the initiation of neovascularisation (McCormick and Zetter, 1992; Stetler-Stevenson et al, 1993).

### *Invasion Events*

Destruction of natural tissue barriers consisting of interstitial connective tissue and basement membrane represents the first step in tumour invasion and permits the local and regional movement of the tumour cells (Tryggvason et al, 1987). The basement membrane is involved in compartmentalisation and also regulates many cellular functions. The major components of the basement membrane include type IV collagen, laminin, heparan sulphate proteoglycan, and destruction of these components correlates with invasion and metastasis of the tumour cells. This step requires the coordinated expression of destructive enzymes

and their inhibitors of which there are 4 main classes namely; serine proteinases, matrix metalloproteinases (MMPs), cysteine proteinases and aspartyl proteinases which allow the tumour cells to escape from the primary mass, and undergo intra and extravasation from the vasculature. MMPs comprise of type IV collagenases named MMP-2 and 9 (gelatinase A and B respectively); interstitial collagenases (MMP-1 and 8) and the stromelysins, namely MMP-3, 10, 11 and 7 (stromelysin-1, 2, 3 and matrilysin respectively). As well as degrading a wide variety of ECM components, the MMPs, specifically MMP-7 and the recently discovered MMP-14, can activate procollagenase and progelatinase respectively, and the activity of the MMPs has been correlated with the ability of a tumour cell to metastasise.

The serine proteinases include plasmin, cathepsin G, thrombin, trypsin, uPA and tPA. uPA and tPA are two of the most well documented serine proteinases, and convert plasminogen into plasmin which can degrade a wide variety of ECM components. uPA binds to a cell surface receptor known as the uPAR which plays a pivotal role in the migration of tumour cells, and both uPA and uPAR have been positively correlated with tumour cell invasiveness. tPA is thought to play a less dominant role in tumour progression than uPA since most tumour cells lack expression of tPA.

The cysteine proteinases include cathepsin B and L which have cysteine residues at their active sites and have been implicated in metastasis (Foekens et al, 1998), as has cathepsin D, which belongs to the aspartyl proteinases (Solomayer EF, 1998).

### *Migration of the tumour cells*

An important step following destruction of the basement membrane is the migration of the tumour cell through membrane barriers. This process is stimulated by factors secreted from the tumour cells or from host stromal cells such as autocrine motility factor (AMF) or hepatocyte growth factor (HGF).

### *Evasion of Immunosurveillance*

Once in the lymph or blood vessels, tumour cells must escape destruction by either immunosurveillance or blood turbulence. During transport, tumour cells can travel individually as single cells or as emboli composed of tumour cells (homotypic) or tumour/



host cells (heterotypic). Most of the tumour cells which leave the primary mass will be destroyed (Weiss L, 1990) leaving as few as one in 10000 of the tumour cells to initiate a new tumour colony at a secondary site (Fidler IJ, 1973). An effective immune response capable of eliminating tumour cells is provided by cytotoxic T cells (CTL) which recognise cell surface epitopes associated with major histocompatibility complex (MHC) class 1 antigens present on the tumour cell surface. The natural killer (NK) cell plays a significant role in the control of metastasis where low levels of MHC class 1 leads to elevated NK sensitivity (Karre et al, 1986). Other tumour cell surface molecules such as ICAM-1 and LFA-3 have been implicated in the cytotoxic response towards tumour cells by interacting with LFA-1 and LFA-2 respectively on the lymphocyte surface. Soluble forms of ICAM-1 have been shown to interfere with the immune targeting of tumour cells by binding to the lymphocyte aiding in the escape of the tumour cells from the immune system. Antibodies secreted by B-lymphocytes (plasma cells) directed towards specific tumour antigens also function to eliminate tumour cells.

#### *Arrest and Organ Colonisation*

In the majority of cases, a metastasising cell passes through the heart before finding a resting place by adhering to a vessel lining. Because the heart pumps all the blood through the lung capillaries, the lungs are often a common site for metastasis. Liver metastasis often arises from colon cancers because the liver receives the direct drainage of venous blood from the large intestine. Nevertheless, simply lodging of tumour cells into an organ due to the emboli being too large to traverse a capillary lumen is insufficient for metastasis to develop (Rusciano and Burger, 1992), and instead, requires a favourable environment for successful organ colonisation. This will depend on a number of factors including the appropriate growth factors and hormones to stimulate the tumour cells (Rusciano et al, 1992), and the growth of new blood vessels. Tumour cells may also arrest in specific organs by exhibiting different capacities to attach to endothelial cells regulated by various chemotactic factors (Lichtner et al, 1989).

### *Angiogenesis*

The formation of a blood supply is a complex process requiring dissolution of the basement membrane, endothelial cell migration and proliferation, vascular formation and development of a new basement membrane. Degradation of the basement membrane by proteolytic enzymes such as MMPs and uPA is essential for successful angiogenesis to occur. Factors such as platelet derived growth factor (PDGF), HGF, and  $\text{TNF}\alpha$  stimulate the formation of new tumour blood vessels, and integrins such as  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$  and  $\beta_1$  also play a role in angiogenesis as they are required for vascular proliferation.

### **1.3 Cell-Extracellular Matrix Interactions**

Disruption of the cell-ECM associations is essential for successful metastasis of the tumour cells allowing changes in the adhesive interactions to occur between tumour cells and the surrounding ECM. The ECM is a complex network of collagenous proteins, glycoproteins, proteoglycans and glycosaminoglycans, and provides a supportive scaffold for cells as well as mediating a variety of cellular processes such as cell attachment, migration and differentiation.

#### *Collagens*

Although the primary role of the collagens is structural, they are also involved in cell attachment and differentiation, and can also function as chemotactic agents and as antigens in immunopathological processes.

There exists greater than 20 collagens which form the major constituents of the ECM. All have domains with a triple helical conformation where each domain has 3 subunits known as  $\alpha$  chains. The  $\alpha$  chains each contain a (Gly-X-Y)<sub>n</sub> repetitive sequence motif where X and Y can be any amino acid but are usually proline or hydroxyproline which stabilises the helical structure.

#### *Type IV collagen*

Type IV collagen is found in the basement membrane and can form a network structure interacting with laminin, nidogen, and heparan sulphate proteoglycan. It exists as heterotrimers of two  $\alpha_1$  and one  $\alpha_2$  chain where separate molecules associate end to end. The procollagen form of the molecule is incorporated directly into the basement membrane structure without undergoing extracellular processing. Collagen type IV has a major cell binding site which interacts with the integrin receptors  $\alpha_2\beta_1$  and  $\alpha_1\beta_1$  (Vandenberg et al, 1991).

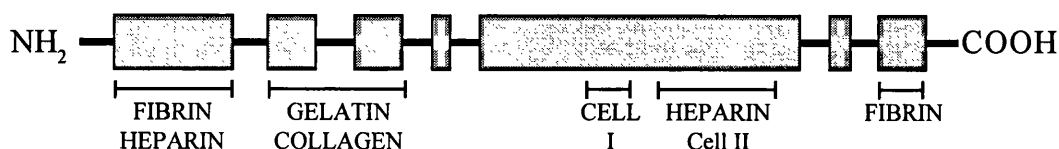
### *Collagen Type I*

Type I collagen belongs to the fibrillar collagens and can be isolated from adult connective tissue such as skin, bone, tendon and cornea. Greater than 95% of the classic fibril-forming collagens are composed of 3 helical chains which wrap around one another to produce the tight triple helical structure of the molecule. Each molecule of type I collagen consists of two  $\alpha 1$  chains and one  $\alpha 2$  chain which are stabilised by interchain hydrogen bonds. The peptide bonds linking adjacent amino acids are buried within the interior of the molecule making the triple helical region highly resistant to attack. Fibrillar collagens can interact with cells directly via specific cell surface receptors or indirectly via other ECM components. A specific region of Type 1 collagen termed the DGEA site has been shown to block the breast adenocarcinoma cell adhesion to collagen type 1 and laminin (Staaz et al, 1991).

### *Fibronectin*

Fibronectin is a large glycoprotein which can be secreted by a wide variety of cell types and is also present in plasma, loose connective tissue and embryonic (but often not adult) basement membranes. It mediates a variety of processes including cell adhesion, embryonic cell migration, wound healing, differentiation and cytoskeletal organisation.

A number of variant forms exist due to alternative splicing of its precursor mRNA (Hirano et al, 1983) and the initial secreted form of fibronectin is composed of a dimer of two subunits held together by a pair of disulphide bonds near the C-terminal. In the ECM, fibrils of fibronectins are further disulphide bonded into high molecular weight polymers. The structure of fibronectin comprises of three general types of homologous repeating units termed I, II, III, of which there are 12 type I repeats (45 amino acids long), two 60 amino acid type I repeats and 15-17 type III repeats each 90 amino acids long.



**Figure 1.** Structure of fibronectin

Specialised domains arise from the repeating units which can bind to specific cell surface receptors or to collagens, proteoglycans or other ECM molecules. Type I forming domains bind heparin, fibrin or collagen whereas the major collagen binding domain comprises largely of Type II repeats which may not actually be essential for the binding of collagen (Ingham et al, 1989). Type III repeats bind to cells or to heparin but not to collagen and most cells adhere to fibronectin at the cell binding domain containing an RGD recognition motif. This RGD motif was the first sequence to be reported active in promoting adhesion (Yamada KM, 1991) and the sequences surrounding the RGD motif appear to be important, specifically, the amino terminal site which promotes cell adhesion by acting synergistically with the RGD site (Obara et al, 1988). Experimental metastasis of murine melanoma cells after intravenous injection can be inhibited with monomeric (Humphries et al, 1986; 1988) and multimeric peptides containing this RGD motif (Saiki et al, 1989).

### *Laminin*

Laminin is one of the major components of the basement membrane and is implicated in roles such as cell adhesion, migration and differentiation. Various isoforms exist (laminins 1-6) where the basic structure comprises of three different subunits linked together by disulphide bonds. This cross-like structure of laminin can interact with other basement membrane components including type IV collagen, heparan sulphate proteoglycan and nidogen.

Laminin contains multiple active sites (Beck et al, 1990) and synthetic peptides comprising of the central region of laminin have been shown to block experimental metastasis (Kleinman et al, 1989). The YIGSR sequence located on the B1 chain can inhibit tumour growth (Fridman et al, 1990; Iwamoto et al, 1987) and metastasis by reducing tumour cell adhesion to laminin, and also inhibits angiogenesis (Sakamoto et al, 1991; Daneker et al, 1989). This peptide has also been found to interact with normal cells and influence cell differentiation (Kleinman et al, 1987). Two other peptides located on the B1 chain, namely PDSGR and RYVVLPR also inhibit tumour metastasis (Murata et al, 1989; Sakamoto et al, 1991; Skubitz et al, 1990). In contrast, a different sequence of laminin known as the PA22-2 protein increases tumour metastasis, growth, angiogenesis, plasminogen activation and collagenase IV activity (Kanemoto et al, 1990; Stack et al, 1990).

At least 6 integrin receptors bind to laminin where the major site of interaction of these

receptors is located at the end of the long arm and near the centre of the laminin cross.

### *Integrin receptors*

There are a number of families of adhesion molecules involved in cell matrix interactions of which the largest and probably most significant are the integrin receptors. As well as providing anchorage to the ECM, integrins are involved in intracellular signalling and have been shown to regulate gene expression affecting processes such as cell migration, proliferation, differentiation and apoptosis (Juliano and Haskill, 1993). They are expressed by most cell types and consist of one  $\alpha$  chain non-covalently associated with one  $\beta$  chain.

### *Role of integrin receptors in tumour progression*

There have been several studies to illustrate the role of certain integrin receptors in tumour progression where changes in expression of the integrin profile are associated with tumour progression. Although the fibronectin receptor  $\alpha_5\beta_1$  and the vitronectin receptor  $\alpha_v\beta_3$  are not expressed in benign melanocyte lesions and early primary melanoma, they emerge in advanced primary melanomas and metastases, and induce enhanced expression of metalloproteases which results in an increase in tumour invasion through matrigel (Seftor et al, 1992; 1993). The collagen receptor  $\alpha_2\beta_1$  is more strongly expressed in invasive melanoma compared to in situ melanoma (Van Duinen et al, 1994) and this reflects in vitro studies where  $\alpha_2\beta_1$  is associated with enhanced invasion of melanoma cell lines (Klein et al, 1991). The  $\alpha_3\beta_1$  receptor for fibronectin, laminin and collagen is expressed by greater than 80% of metastatic melanomas and enhanced expression on tumour cells correlates with dermal invasiveness (Natali et al, 1993). Expression of the  $\alpha_4\beta_1$  integrin which binds to fibronectin and  $\alpha_v\beta_3$  (Delannet et al, 1994) are significantly increased in metastatic melanomas (Schadendorf et al, 1993).

In contrast,  $\alpha_5\beta_1$  has been shown to have a tumour suppressor effect in chinese hamster ovary cells whereby clones overexpressing  $\alpha_5\beta_1$  were shown to grow less rapidly in nude mice compared to clones lacking the fibronectin receptor (Schreiner et al, 1989). Melanoma cells expressing  $\alpha_v\beta_3$  have been found to grow more slowly in nude mice compared to clones expressing high levels of this receptor (Felding-Habermann et al, 1992).

#### **1.4. Involvement of the plasminogen activation system in tumour invasion and metastasis**

Once a tumour cell has adhered to the ECM, it must invade the surrounding basement membrane in order to proceed with the metastatic process. Tumour cell invasion requires the localised expression of a number of proteolytic enzymes which facilitate invasion of the tumour cells through surrounding tissues (Liotta et al, 1991; Mignatti and Rifkin, 1993), and the plasminogen activation system has been shown to play a pivotal role in such tumour cell invasion (Dano et al, 1985). Plasminogen activators (PAs) mediate the conversion of the inactive zymogen plasminogen into the active component, plasmin, which has a broad substrate specificity, and which is inhibited by  $\alpha_2$  macroglobulin and  $\alpha_2$  anti-plasmin. Plasmin can convert the precursors of growth factors such as TGF $\beta$  to the biologically active form (Flaumenhaft et al, 1992) and is also a key player in degrading a wide variety of ECM components (Dano et al, 1985) such as fibronectin, laminin, type IV collagen and tetranectin. As well as ECM degradation, plasmin can also activate procollagenases which play an important role in tumour cell invasion (Meissauer et al, 1992). Two well documented activators of plasminogen are uPA and tPA.

##### *Urokinase type plasminogen activator*

The human 6.4kb uPA gene which is located on chromosome 10, contains 11 exons and codes for a 53kDa protein which acts as a fibrin-independent, largely cell surface receptor-bound plasminogen activator. Melanoma cells can utilise uPA for both matrix degradation (Quax et al, 1991; Stahl and Mueller, 1994) and invasion (Stahl and Mueller, 1994; Supino et al, 1992). The importance of uPA in the metastatic spread of tumour cells has previously been demonstrated by the addition of anti-uPA antibody which has been shown to inhibit the metastatic spread of mouse B16F10 melanoma cells (Hearing et al, 1988). Further supporting evidence for a role of uPA in metastasis has been demonstrated using uPA-negative B16F1 cells which, when transfected with sense human uPA, results in a more metastatic phenotype, and likewise, the antisense transfection of uPA in uPA-producing B16F10 cells results in a less metastatic phenotype (Yu and Schultz, 1988). Synthetic peptides comprising of the binding domain of uPA are able to inhibit spontaneous metastasis in the nude mouse (Kobayashi et al, 1994). The invasive properties of tumour cells and

their ability to degrade the ECM can be reduced when pretreated with anti-uPA antibody (Mignatti et al, 1986; Reiter et al, 1993). As well as having a role in tumour invasion, uPA can also act as an (autocrine) mitogen, as inhibition of uPA-binding has been shown to inhibit cell proliferation (Kirchheimer et al, 1989). uPA is also thought to play a dominant role in tissue remodelling processes such as wound healing (Grondahl-Hansen et al, 1988), monocyte invasion (Kirchheimer and Remold, 1989) and angiogenesis (Bacharach et al, 1992). Tumour progression is accompanied by the loss of differentiation markers and gain of progression markers like uPA, and Supino et al, (1992; 1994) found an inverse relationship between melanin production and uPA production where invasiveness was correlated with uPA production and inversely correlated with melanin content. The correlation of uPA with invasiveness and metastatic potential has been exploited in the clinical situation where it has been found that in breast carcinomas, high levels of uPA are associated with poor prognosis (Janicke et al, 1990; Grondahl-Hansen et al, 1993).

#### *uPA receptor*

uPA binds with high affinity to a 55-60kDa GPI-linked membrane receptor known as the uPAR (Ploug et al, 1991) via its amino terminal domain. uPA exists as both a single and a two chain form, however for the single chain form (pro-uPA) to become active, it must bind to the uPAR (Blasi F, 1988). This interaction, which occurs within the proximity of receptor bound plasmin, accelerates the conversion of pro uPA into active uPA (Quax et al, 1991). uPA and uPAR play a diverse role in processes mediating cell migration and homing during embryogenesis, inflammation, angiogenesis, wound healing, tumour cell invasion and metastasis (Vassalli et al, 1991) and have been identified as markers of melanoma progression (de Vries et al, 1994; Delbaldo et al, 1994). uPA bound to the uPAR is a key step in the migration of tumour cells (Bruckner et al, 1992) and monocytes (Kirchheimer et al, 1989b), and can be correlated with the invasive capacity of the tumour cell (Will et al, 1994). Both uPA and its receptor are expressed at focal cell-cell or cell-substrate adhesion sites, and in cultured keratinocytes, uPA colocalises with E-cadherin (Jensen and Wheelock, 1992). In migrating cells, uPAR is concentrated at the ruffled edge of cells and in the proximity of focal adhesion sites. The uPAR is found to associate with the  $\beta 2$  integrin family of adhesion molecules (Bohuslav et al, 1995) and also with the  $\alpha \beta_3$  integrin which colocalises with the uPAR in cultured glioblastomas (Gladson et al,



1995) and in metastatic melanoma cells (Nip et al, 1995). Unoccupied uPAR can also function as an adhesion molecule for vitronectin (Waltz and Chapman, 1994) suggesting a role for this receptor in adhesive processes as well as invasion. The regulation of uPAR synthesis is thought to involve PKC which has been implicated in post adhesion, integrin-mediated signalling and the inhibition of PKC was shown to block cell spreading as well as the phosphorylation of focal adhesion kinase, pp125FAK (Shattil et al, 1994).

The importance of the uPAR in metastasis can be demonstrated by blocking this receptor with anti-uPAR antibody which abolishes cell surface plasminogen activation (Ronne et al, 1991) making cells less invasive in matrigel (Mohanam et al, 1993). Pro-uPA can compete with the soluble form of uPAR thereby preventing pro-uPA from binding to its receptor which can also inhibit invasion (Wilhelm et al, 1994).

#### *Tissue type plasminogen activator*

The gene for tPA is located on chromosome 8 and consists of 14 exons encoding a 70kDa protein. tPA acts as the major circulating activator which binds to and is activated by fibrin. tPA plays a role in fibrinolysis and long term memory (Qian et al, 1993) and can activate plasminogen in the adult brain (Sappino et al, 1993). Levels of this PA can be induced by matrix fragments as demonstrated by Stack et al, (1993) who showed a 10-fold increase of tPA activity in conditioned medium of B16F10 cells incubated with laminin A fragments. Expression of tPA is thought to play a less dominant role in metastasis than uPA as t-PA levels are almost undetectable in many human tumours. However, the exception appears to be melanoma cells which have been shown to express higher levels of tPA than most other tumour cell types, and are therefore often the source for large scale purification of tPA (Collen et al, 1982). High production of tPA by tumour cell lines is apparently unique for melanoma and neuroblastoma cell lines (Vaheri et al, 1994) and this appears to be typical for neural crest-derived cells. In vitro primary cultures of melanocytes also produce tPA (Hashimoto et al, 1986), but despite this finding, no tPA has been detected from melanocytes either single or grouped as naevi in normal skin.

The expression of tPA from melanoma cells has been correlated with the ability of these tumour cells to invade a reconstituted matrix and degrade the ECM (Meissauer et al, 1992) and the use of anti-tPA antibodies inhibits migration of the tumour cells through a keratinocyte layer (Meissauer et al, 1991). Invasion of the mouse melanoma cell line, M2

can also be reduced by addition of tPA antibodies to the upper chamber of a Boyden chamber assay (Reich et al, 1988) whereas, in contrast, Mignatti et al, (1986) showed there was no inhibitory effect of anti-tPA antibodies on invasion of B16 cells. Xenografts of non-uPA producing non-metastatic melanoma cells contained approx 8 times more tPA than xenografts of moderately metastatic cells (Quax et al, 1991) supporting the lack of a role for tPA in metastasis. In contrast, uveal lesions of melanoma with a poor prognosis produce higher levels of tPA (De Vries et al, 1995) and tPA transfected melanoma cells injected into the eyes of mice give rise to a higher frequency of metastasis (Alizadeh et al, 1995) which can be inhibited by anti-tPA antibodies.

### *Plasminogen activator inhibitors*

Several plasminogen activator inhibitors exist namely, PAI-1, PAI-2, protease nexin 1 and PAI-3 (Kruithof EK, 1988) which belong to the group of serpins (serine protease inhibitors). In nude mouse xenografts of PAI-1 positive melanoma cells and in human melanomas, the tumour cells make the mRNA for PAI-1 but protein is deposited in the ECM and can associate with vitronectin in the ECM (Seiffert et al, 1990; Hagege et al, 1992). Both PAI-1 and PAI-2 are capable of inhibiting tPA and uPA but do not bind to pro uPA (Andreasen et al, 1986). The complex of uPA/PAI-1 is recognised by the LDL receptor-related protein, LRP (also known as  $\alpha 2$  macroglobulin receptor) and can be internalised via a clathrin coat pit mechanism. Following PAI-1 binding, the uPAR:uPA:PAI forms a complex with the 515kDa chain of LRP. uPA:PAI-1 are targeted to the lysosomes for eventual degradation and the uPAR and LRP are recycled and redistributed at new focal adhesion sites on the cell surface (Herz et al, 1992). Unlike PAI-1, PAI-2 is not secreted into the extracellular matrix and is localised mainly in the cytoplasm (Berlin et al, 1989). It would be expected that high levels of PAI-1 would be beneficial by inactivating uPA. However, in breast cancers, high levels of PAI-1 are associated with worse prognosis (Grondahl-Hansen et al, 1993) and expression of PAI-1 correlates with metastatic capability in nude mice (Quax et al, 1991). In contrast, melanoma cells transfected with PAI-2 give rise to significantly less metastasis in nude mice and in vitro, PAI-1 or 2 effectively inhibits matrix degradation (Reiter et al, 1993).

## **1.5. Tumour cell evasion of the immune system**

During metastasis, the tumour cells have to evade immunosurveillance by cells of the immune system. The immune system comprises of various subsets of lymphocytes, often referred to as effector cells, where specific cell types have the ability to lyse target tumour cells. B and T lymphocytes are the principle mediators of an effective immune response and are under the control of dendritic cells (reviewed in Banchereau and Steinman, 1998). Dendritic cells are located in most tissues and capture and process antigens then display them in their major histocompatibility complex (MHC) molecules. They then migrate to the lymphoid organs where they can activate T lymphocytes which in turn, induces B cell growth and antibody production directed towards specific antigens. T lymphocytes express receptors (TcR) which recognise processed antigen in the context of MHC on the surface of antigen-presenting cells, and cytotoxic T cells and helper T cells recognise MHC class I and class II molecules respectively.

In tumour immunosurveillance, several cell surface molecules play a pivotal role in the recognition and binding of the tumour cells to lymphocytes, and these include MHC class I and ICAM-1.

### **1.5.1. Major histocompatibility complex class-1**

MHC class-1 molecules, also known in humans as human leucocyte antigen ABC (HLA-ABC), are cell surface polypeptide structures which have short hydrophobic segments spanning the membrane. The complex consists of 2 chains, namely the  $\alpha$  (or heavy) chain which is approximately 44kDa, and the  $\beta$  chain (12kDa). The principle function of the HLA-ABC molecule is to bind fragments of foreign proteins which can be presented and recognised by the lymphocytic cells. A subpopulation of lymphocytes termed natural killer cells (NK cells) express inhibitory receptors which recognise the presence of the HLA-ABC complex, and in doing so, inhibit lysis of the tumour cell (Kaufman et al, 1995; Litwin et al, 1994). In malignant transformation, HLA-ABC levels are often reduced therefore, the NK cells do not receive the signals and target cell lysis occurs (Mingari et al, 1997). T cells also express these inhibitory receptors which can block the T-cell mediated activation of cytotoxic T lymphocytes. As well as the role of the MHC class 1 complex in tumour cell lysis, the molecular mechanisms behind this lytic process require the binding

of tumour cells to the effector cells, and ICAM-1 expression is critical for this interaction.

### **1.5.2. Intercellular adhesion molecule-1**

The transmembrane glycoprotein, ICAM-1 (CD54) is a member of the immunoglobulin superfamily (Staunton et al, 1988) whose basic function is the induction of specific and reversible cell-cell adhesion. Other members of this group include ICAM-2, ICAM-3 (CD45), neural cell adhesion molecule (NCAM), vascular cell adhesion molecule (VCAM) and platelet endothelial adhesion molecule (PECAM).

#### *ICAM Receptors*

All 3 ICAM members bind to 2 integrin receptors namely leucocyte function associated receptor (LFA-1; CD11a/CD18) (Marlin and Springer, 1987; Binnerts et al, 1994) and Mac-1 (CD11b/CD18) (Diamond et al, 1990) which both belong to the  $\beta 2$  integrin subfamily and are expressed by leucocytes (Springer TA, 1990). ICAM-1 exhibits the highest and ICAM-3 the lowest affinity for LFA-1. Both LFA-1 and Mac-1 require activation to expose a high affinity ICAM-1 binding epitope which is dependent on the presence of divalent cations. This results in signalling pathways involving activation of PKC and increases in intracellular calcium concentrations, and is inhibited by rises in the levels of cAMP. Only a small population of Mac-1 binds effectively to ICAM-1 (Diamond et al, 1991), but the structure of ICAM-1 probably allows both LFA-1 and Mac-1 to bind to ICAM-1 simultaneously (Staunton et al, 1988). Activation of LFA-1 by binding to ICAM-3 on leucocytes can enhance the avidity of LFA-1 for ICAM-1 which is possibly mediated by the phosphorylation of the  $\beta$  chain of LFA-1 resulting in a conformational change in the molecule. Although LFA-1 is probably the most important ligand for ICAM-1 in vivo since it is highly conserved between species, other less well characterised receptors for ICAMs exist. One such receptor is sialophorin (CD43), which is abundantly expressed by leucocytes and platelets (Rosenstein et al, 1991), although its role in adhesion is unclear. ICAM-1 also serves as a receptor for soluble fibrinogen and hyaluronan (Languino et al, 1993; McCourt et al, 1994). The binding of soluble fibrinogen requires the clustering of ICAM-1 as has previously been demonstrated to occur on endothelial cell projections

(Stolpe et al, 1993) and also requires the presence of divalent cations. Human ICAM-1 also serves as a receptor for the major group of rhinoviruses (Greve et al, 1989) and an additional epitope within the first domain of human ICAM-1 has been characterised as the binding site for *Plasmodium falciparum*-infected erythrocytes.

### *ICAM Gene and Protein*

The human ICAM-1 and ICAM-3 genes map very close together on chromosome 19p13.2-13.3 (Bossy et al, 1994) whereas the human ICAM-2 gene maps to chromosome 17q23-25 (Sansom et al, 1991). The human ICAM-1 gene consists of seven exons separated by six introns which encodes 5 Ig-like domains, as is similar for ICAM-3 (Fawcett et al, 1992), whereas, ICAM-2 has only 2 Ig-like domains (Staunton et al, 1989). Each of the 5 Ig-like domains is encoded by a separate exon (Voraberger et al, 1991). Human ICAM-1 cDNA appears to have a limited homology (55-65%) with that of murine, rat and canine cDNA. The ICAM-1 protein comprises of a total of 505 amino acids and 453 of these amino acids, most of which are predominantly hydrophobic, form the Ig-like domains. The hydrophobic transmembrane domain is made up of 24 residues and the other 28 amino acids form the charged cytoplasmic tail (Staunton et al, 1988).

The extracellular part of ICAM forms a hinged rod with length of approx 19nm and width 2-3nm suggesting the possibility of end to end alignments of the Ig domains (Staunton et al, 1990; Giranda et al, 1990). Free cysteine residues are located close to the membrane which may allow formation of intermolecular disulphide bonds suggesting the possibility of dimer formation (Staunton et al, 1988). The amino terminal first Ig-like domain of ICAM-1 is responsible for binding to LFA-1 (Staunton et al, 1990) whereas on LFA-1, the ICAM-1 binding site is located on domains 5 and 6 of the CD11a subunit (Stanley et al, 1994). The ICAM-1 protein does not contain a calcium-binding RGD sequence suggesting its conformation is not dependent on the presence of calcium, unlike the usual ligands for receptors of the integrin family (Staunton et al, 1990).

### *Glycosylation of ICAM-1*

The molecular weight of ICAM-1 varies and is dependent on the differential glycosylation state of the molecule, which is in turn, dependent on the cell type giving rise to molecular

weights between 80 and 114kDa (Rothlein et al, 1986). Deglycosylation of the molecule can also occur and results in a protein of 60 kDa (Dustin et al, 1986). Glycosylation may act as a mechanism of affinity regulation as N-linked glycosylation has been reported to reduce affinity of ICAM-1 for Mac-1 whereas, in contrast, glycosylation seems to have no effect on binding of LFA-1, rhinovirus or malaria-infected erythrocytes. Differences in the actual activity of glycosylation enzymes adds further to the cell specificity of ICAM-1 protein expression.

### *Cytoskeletal association and signalling role of the ICAMs*

Association of ICAM-1 with the cytoskeleton may induce changes in ICAM-1 conformation and ligand affinity. ICAM-1 is distributed in the membrane in a polarised fashion and can associate and colocalise with  $\alpha$ -actinin (a cytoskeleton binding protein) (Carpen et al, 1992). The cytoplasmic tails are the most divergent regions of the molecules suggesting that the ICAMs might have evolved to develop different signalling or cytoskeletal roles. No evidence exists for proteins interacting with the cytoplasmic tail of ICAM-2. However there does appear to be a role for ICAM-3 in a number of signalling events. ICAM-3 has been shown to transduce signals into the cell and to induce changes in intracellular calcium levels and tyrosine phosphorylation and can also signal to LFA-1 to upregulate binding to ICAM-1 (Hernandez et al, 1993). Signalling via ICAM-1 is also thought to occur as cross-linking of ICAM-1 with antibodies leads to the inhibition of IFN $\gamma$ , TNF $\alpha$  and IL-1 from lymphocytes. In brain endothelial cells, activation of the kinase pp60src occurs during ICAM-1 cross-linking and is associated with phosphorylation of target proteins including the cytoskeletal binding protein, cortactin.

### *Regulation of ICAM-1*

ICAM-1 is expressed in a wide variety of cell types including endothelial, epithelial, macrophages, fibroblast-like cells and dendritic cells in the human thymus, lymph nodes, intestine, skin, kidney and liver. In hemopoietic cells, ICAM-1 is also expressed on bone marrow progenitors and on cells of the lymphoid and myeloid lineage. The expression of ICAM-1 is regulated predominantly at the transcription level where Northern blot analysis reveals the presence of 2 transcripts, although their significance is unclear as they do not

give rise to 2 different proteins, nor are they the result of alternative splicing or differential usage of transcription initiation sites (Voraberger et al, 1991; Staunton et al 1988). Expression of ICAM-1 can be induced or up regulated by cytokines such as TNF $\alpha$ , IFN $\gamma$ , IL-1 and IL-6, whereas the glucocorticoids (Stolpe et al, 1993) act as inhibitory agents of ICAM-1 expression. The 5' region flanking the human ICAM-1 gene contains 3 transcription initiation sites (Degitz et al, 1991) which may be used differentially depending on cell type and stimulus (Voraberger et al, 1991). IL-6 and IFN $\gamma$  (Caldenhoven et al, 1994; Jahnke and Johnson, 1994) regulate ICAM-1 transcription through a shared palindromic sequence designed pIRE. Both IL-6 and IFN $\gamma$  induce binding of the same transcription factor, probably related to the p91 signal transducer and activator of transcription 1 (STAT 1 or IFN $\gamma$  activating factor), while in addition, IL6 induces binding of signal transducer and activator of transcription 3 (STAT-3). The  $\kappa$ B enhancer in the ICAM promoter conveys responsiveness to TNF $\alpha$ , IL-1 and lipopolysaccharide (Ledebur and Parks, 1995). This enhancer binds a complex of transcription factors of RelA or Rel A/c-Rel in addition to the nuclear factor (NF)  $\kappa$ B transcription factor (NF $\kappa$ B1-RelA) (Parry and Mackman, 1994). The glucocorticoid receptor physically interacts specifically with the RelA subunit of the NF $\kappa$ B complex causing inactivation of the transcription factor (Caldenhoven et al, 1995).

The first intron of ICAM-1 has other putative enhancer elements which include 2  $\kappa$ B-like elements and an Sp1 sequence. Sp1 sites probably regulate constitutive ICAM-1 transcription as elimination of the Sp1 site results in a drop in basal promoter activity.

The AP-1 proteins, jun and fos act synergistically with NF $\kappa$ B to transactivate a  $\kappa$ B enhancer element or an AP-1 site (Stein et al, 1993) to modulate ICAM-1 transcription. The AP-2-like site in the ICAM-1 promoter has been shown to mediate the UVA but not UVB effects on ICAM-1 transcription (Grether-Beck et al, 1995).

Although transcriptional regulation of ICAM-1 accounts for most regulatory effects of ICAM-1, IFN $\gamma$  may also upregulate ICAM-1 expression by stabilising mRNA. The 3' untranslated region of ICAM-1 mRNA contains multiple destabilising AUUUA sequences which act as targets for the mRNA stabilising agents (Ohh et al, 1994).

### *In Vivo Mouse Model of ICAM-1*

The importance of ICAM-1 in vivo has been demonstrated using mice which have an inactivated gene for ICAM-1 (by homologous recombination in embryonal stem cells). The animals develop normally and are viable. However, blood neutrophil counts are increased (Xu et al, 1994) and activation and migration of leukocytes to places of inflammation is reduced (Sligh et al, 1993). The absence of ICAM-1 seems to protect mice from lethal septic shock induced by bacterial LPS by reducing leukocyte-endothelial cell interactions (Xu et al, 1994).



### **1.5.3. Soluble intercellular adhesion molecule-1**

One possible mechanism as to how tumour cells evade immunosurveillance by lymphocytes is through the release of a soluble form of ICAM-1 (sICAM-1) which may exist as a multimeric form or complexed with other proteins due to the existence of various isoforms (240, 430, and >500kDa) (Seth et al, 1991). sICAM-1 is of lower molecular mass (80kDa) (Rothlein et al, 1991) than membrane ICAM-1 (90kDa) (Marlin and Springer, 1987) and is similar to recombinant sICAM-1 comprising of the extracellular part of ICAM-1. Glycosylation of sICAM-1 can occur, which is cell type-specific and may also contribute to variations in molecular weight. Mononuclear cells, endothelial cells, keratinocytes, hepatocytes and melanoma cells are sources of sICAM-1 which is also present in plasma, cerebrospinal fluid and sputum. The expression of sICAM-1, like mICAM-1, is also regulated by IL-1, TNF $\alpha$ , IFN $\gamma$  which have been shown to induce increased shedding of sICAM from melanoma cells (Becker et al, 1991), yet IL-6 which alters mICAM-1 expression, is unable to induce this shedding. Serum levels of sICAM-1 have been reported to increase in various disease states (Banks et al, 1993) which include human malignant melanoma (Harning et al, 1991), but the biological consequences of such increases remains controversial. Serum levels are also increased in patients with leucocyte adhesion deficiency who lack the  $\beta 2$  integrin chain of LFA-1 (Rothlein et al, 1991) due either to increased ICAM expression associated with chronic infection or to the absence of functional LFA-1 and Mac-1 molecules to bind sICAM. Expression is also elevated in patients with renal failure, non-Hodgkin lymphoma, ovarian and breast carcinoma, gastrointestinal tumours, renal and bladder carcinoma. It is possible that binding of sICAM-1 to the LFA-1 receptor could prevent the lymphocyte from interacting with mICAM-1 and thus prevent tumour cell lysis occurring. This is supported by reports from various groups (Altomonte et al, 1993; Becker et al, 1992) but which is in contrast to the recent data from Young et al (1997) who reported on the inability of sICAM-1 to affect tumour cell lysis. Monomeric sICAM containing only the first 2 N-terminal domains is able to bind malarial-infected erythrocytes, and sICAM-1 also inhibits cellular infection by a major group rhinovirus (HRV54) and coxsackie A13 virus (Crump et al, 1993; Marlin et al, 1990) which both use ICAM-1 as their membrane receptor. sICAM-1 has also been reported to inhibit adhesion of monocytic cells to purified ICAM-1 (Welder et al, 1993).

## **1.6. Why study melanoma?**

Melanoma is one of the most aggressive cancers known and in the UK, is the 4th most common cancer in women and the seventh commonest in men in the 15-35 age group. Melanocytes originating from neural crest cells located within the basal layer of the epidermis closely interact with surrounding basal keratinocytes to form a functional structure defined as the epidermal-melanin unit. Melanocytes protect keratinocytes against UV damage through the synthesis of melanin pigment and the transfer of melanosomes to adjacent basal keratinocytes by long dendritic processes. Melanoma is derived from these melanocytes, and sun exposure which induces irreversible DNA damage, is the major causal factor for skin cancer.

The incidence of cutaneous melanoma has steadily increased worldwide with an increase from 3.5 to 7.8 cases per 100 000 per year in men and from 6.8 to 12.3 per 100 000 per year in women during the period of 1979-1994. In 1979, deaths occurring due to cutaneous malignant melanoma rose in men from 1.3 per million per annum to 2.3 per million per annum by 1994. The figures for women show that in 1979, 2.4 deaths occurred per million per annum which fell to 1.9 million per annum in 1994 (MacKie et al, 1997). Survival rates depend on the depth of invasion of the primary tumour and subsequent involvement of regional and distant lymph nodes and the overall 5 year melanoma-free survival rate in men is currently 69% and 82% in women (MacKie et al, 1997). Non-invasive, benign lesions are limited to the epidermis with progression occurring to the papillary dermis followed by invasion of the adjacent reticular dermis and subcutaneous fat cells. The spread of melanoma cells into the bloodstream can be detected at very early stages of the disease (Brossart et al, 1993) and high mortality rates occur due to the widespread metastasis of melanoma to sites including the liver, lungs, bone, brain, adrenal glands and heart (Herlyn M, 1990; Clark et al, 1986).

Surgical excision of localised melanoma lesions generally results in a high cure rate (Morton et al, 1993) but no curative therapy is currently available for advanced melanomas. Therefore, it is important to gain a better understanding of melanoma metastasis to allow further insight into the basics of tumour biology which may lead to the development of more effective therapies.

### **1.7. The use of retinoic acid and pentoxifylline in tumour biology**

Two drugs which have been exploited for their potential use as anti-tumour agents are the vitamin A metabolite, retinoic acid (RA), and the phosphodiesterase inhibitor pentoxifylline (PX). RA has reached clinical trials having been used as a therapeutic agent to delay, reverse or block cancer development (Greenwald et al, 1995, Karie et al, 1994) and is known to have the ability to modulate various steps of metastasis including inhibiting cancer cell proliferation (Houle et al, 1991) and enhancing apoptosis (Lotan R, 1995). On the otherhand, PX has been shown to enhance chemotherapeutic drugs, improving the cytotoxic effects of alkylating agents in vitro and in vivo (Boike et al, 1990; Tanaka et al, 1991; Teicher et al, 1991) and can significantly enhance cisplatin-induced cytotoxicity directed against human ovarian and cervical cancer cell lines (Boike et al, 1990).

The following two sections give a general overview of either drug explaining their possible mechanisms of action and their effects on a range of biological processes.

## **1.8. Introduction to retinoic acid**

### *Structure of Retinoic Acid*

Retinoic acid (RA) belongs to the family of molecules termed the retinoids which are biologically active and synthetic derivatives of vitamin A. RA is a yellow crystalline polyene carboxylic acid produced from the metabolic oxidation of vit A and is systematically known as 3, 7-dimethyl-9-(2, 6, 6, -trimethyl-1-cyclohexen-1-yl)-1, 4, 6, 8-nonatetraenoic acid. This hydrophobic molecule is insoluble in water and its biological activity can be modified by cis or trans isomerisation to produce 9-cis or all-trans RA. There is evidence that the interconversion of all-trans and 9-cis RA occurs in mammalian cell lines in vitro (Heyman et al, 1992; Allegretto et al, 1993) and all-trans RA can also undergo isomerisation to 13-cis RA (isotretinoin).

### *Vitamin A Deficiency*

Reports dated as far back as 1925 show that vitamin A-deficient animals develop a wide range of abnormalities such as squamous metaplasia in the epithelia of the eye, nasal passage and the respiratory tract (Wolbach and Howe, 1925). Epidemiological studies in humans indicate individuals with a lower dietary vitamin A intake are at a higher risk of developing cancer (Hong et al, 1995).

Since then, it has been shown the retinoids, in particular RA, are essential for growth, differentiation, formation of the nervous system, limb morphogenesis (Goodman DS, 1984), spermatogenesis (Skinner MR, 1991) and the immune system (Smith et al, 1987, Buck et al, 1990).

### *Vitamin A metabolism*

The main source of vit A are dietary, derived from provitamin A carotenoids from vegetables (eg  $\beta$ -carotene) or fatty acid retinyl esters (R-FA) from animal sources or dietary supplements (Olson JA, 1989).  $\beta$ -carotene can either be absorbed intact or cleaved in the intestinal tract to form two molecules of retinaldehyde. Subsequently, retinaldehyde is reduced by an aldehyde reductase to retinol.

R-FA is hydrolysed in the intestinal lumen to free retinol which is absorbed by enterocytes of the gut (Blomhoff et al, 1992). Serum retinol binding protein (RBP) stored and synthesised in the liver (Blaner WS, 1989) acts as a transport system for retinol from the liver to other tissues. Retinol uptake by cells is mediated by membrane receptors for RBP (Bavik et al, 1992) but the molecular mechanisms are not clear. In blood plasma, it is found associated with transthyretin (TTR), a 55kDa protein. When plasma RBP levels decrease, plasma retinol is also decreased (Berni and Formelli, 1992).

### *Cellular Retinoic Acid Binding Proteins*

The intracellular concentration and types of RA metabolites entering the cell appear to be regulated by 4 retinoid-binding proteins, namely cellular retinol binding proteins I and II (CRBP I, II) and the cellular retinoic acid binding proteins (CRABP I, II).

The dietary retinol in the enterocytes is bound by a cellular retinol binding protein (CRBP II), esterified to R-FA by lecithin:retinol acyltransferase (LRAT), and incorporated into lipoprotein particles (also called chylomicrons). In hepatic parenchymal cells, the free retinol interacts with CRBP I (Ross A, 1993) and can either be transferred to newly synthesised RBP for secretion or transferred to neighbouring hepatic stellate cells for esterification and storage in conditions of dietary sufficiency. The rate of retinol uptake from serum RBP decreases in Sertoli cells when available CRBP sites have been occupied (Shingleton et al, 1989). CRBP II, unlike the widely distributed CRBP I is restricted to the small intestine (Chytil and Ong, 1987). CRBP I has been postulated to capture retinol which when bound, serve as substrate for a specific NADP-dependent dehydrogenase and a cytosolic retinal dehydrogenase leading to all-trans RA synthesis (Posch et al, 1992; Napoli et al, 1995).

CRABP 1 can function as a carrier for the catabolism of all-trans RA to more polar metabolites in vitro (Fiorella and Napoli, 1991). Overexpressing CRABP I in the RA-responsive F9 teratocarcinoma cells alters amounts and types of all-trans RA metabolites and reduces the half life of RA (Boylan and Gudas, 1992) resulting in a reduction of expression of RA-responsive genes (Boylan and Gudas, 1991). Embryonic tissues expressing CRABP I are most sensitive to the teratogenic action of RA (Ruberte et al, 1992).

CRABP II is induced by RA (Astrom et al, 1991) and binds all-trans RA (Fiorella and

Napoli, 1991) suggesting it prevents RA from becoming excessive and causing a teratogenic effect (Maden et al, 1988; Giguere V, 1994). CRABPs may also sequester all-trans RA in the cytoplasm and prevent the retinoids from reaching nuclear receptors. Neither CRABP I nor CRABP II bind 9-cis RA (Fogh et al, 1993) suggesting the existence of a distinct metabolic pathway for 9-cis retinoids which is independent of CRBPs or may involve a distinct family of binding proteins. Availability of active retinoids or their precursors is dependent also on the cellular ratios of CRBP and CRABP (Gustafson et al, 1993).

It is unclear how cells which may lack CRBPs would be protected from toxic levels of retinol as CRBP levels have been found not to be the major determinants of retinol uptake and esterification in squamous carcinoma cell lines (Wahlberg et al, 1989).

#### *CRABP Null Mice*

Mice homozygous for a null mutation of CRABP I gene are phenotypically normal, and double mutant mice for CRABP I and CRABP II are also normal (Lampron et al, 1995). This indicates that either CRABP I and II do not play crucial roles in retinoid function (Gorry et al, 1994) or alternatively, the possibility of redundancy with other proteins may exist.

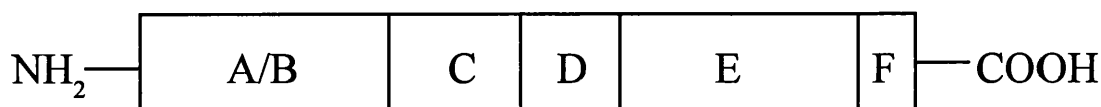
#### **1.8.1. Retinoid mechanisms of action**

The retinoids exert their effects through nuclear receptors belonging to the superfamily of nuclear ligand activated transcriptional regulators, which includes the thyroid hormone receptor (TR) and the vitamin D3 receptor (VDR). The retinoids interact with 2 classes of receptor, namely the retinoic acid receptor (RAR) which binds all-trans and 9-cis RA and the retinoid receptor (RXR) which binds only 9-cis RA (Heyman et al, 1992), although 9-cis RA has a higher affinity for RARs than RXRs (Kersten et al, 1996). Each subclass of receptor can be divided into 3 subtypes namely alpha ( $\alpha$ ) (Petkovich et al, 1987; Giguere et al, 1987; Mangelsdorf et al, 1990), beta ( $\beta$ ) (Brand et al, 1988; Leid et al, 1992; Benbrook et al, 1988) and gamma ( $\gamma$ ) (Krust et al, 1989). Focusing on the RARs, there are 3 major isoforms of  $\alpha$ , seven of the  $\gamma$  subtype, and 4 major isoforms of the  $\beta$  subtype (Dolle et al, 1990).

In humans, each subtype is encoded by a distinct gene where RAR $\alpha$  is mapped to chromosome 17q21.1; RAR $\beta$  to chromosome 3p24 and RAR $\gamma$  to chromosome 12q13 (Mattei et al, 1991). The RXR $\alpha$  subtype in humans maps to 9q34.3; RXR $\beta$  to 6p21.3 whereas RXR $\gamma$  maps to chromosome 1q22-q23 (Almasan et al, 1994).

### *Structure of the receptors*

Both the RARs and the RXRs have conserved structures and their amino acid sequence can be divided into 6 regions termed A-F based upon homology among themselves and with other members of the nuclear receptor superfamily (Leid et al, 1992).



**Figure 2.** Retinoic acid receptor structure

Regions A/B encode transcriptional activation function (AF-1) which modulates target gene transcription and does not require ligand for its activity. The AF-1s show cell-type specificity (Folkers et al, 1993) which suggests they do not interact directly with the basic transcriptional machinery but provide intermediary contacts with cell-type specific proteins. The isoforms for each receptor subtype differ from each other in their A region and these subtypes arise due to alternative splicing and differential usage of 2 promoters, one of which is RA-inducible (Leroy et al, 1992). Transcriptional activity can be enhanced via the phosphorylation of serine residues in the amino terminal domain of the RARs (Huggenvik et al, 1993) and is common for RAR $\gamma$  (Rochette-Egly et al, 1991). Region C marks the DNA-binding domain which regulates target gene expression by recognising specific sites on the target DNA. This region is highly conserved between all 3 RAR subtypes (94-97%). Region D acts as a hinge region between regions C and E, and region E itself contains the ligand-binding domain, the ligand-dependent transcriptional activation function (AF-2) (Nagpal et al, 1993) and the receptor dimerisation surface. RXR AF-2s are unmasked or activated in the presence of 9-cis RA and RAR AF-2s are activated in the presence of 9-cis RA or all-trans RA (Nagpal et al, 1993). Thus binding of the hormone

induces a conformational change that allows the appearance of an activation domain at the surface of the protein. The AF-1s of the A/B domain show weak activation properties by themselves but can strongly synergise with their corresponding AF-2 (Nagpal et al, 1993). The function of the F region remains unknown.

### *Interaction of the receptors with target genes*

The receptors act as ligand-dependent transcription factors which bind to specific sites on the DNA termed the retinoic acid response elements (RARE) (Naar et al, 1991). The RARE sequences are characterised by direct repeats (DR) of (A/G)GGTCA separated by 2 or 5 (DR2 or DR5) nucleotides (Umesono et al, 1991). The RAR and RXR form heterodimeric complexes with one another which bind to the RARE (de The et al, 1990). RXRs can also function on their own in the presence of specific ligands and form homodimers (Zhang et al, 1992a) and can act as coreceptors for thyroid hormone receptors (TRs), vitamin D receptors (VDRs) and other receptors (Yu et al, 1991; Zhang et al, 1992b; Kliewer et al, 1992). The autoinduction of RAR expression occurs due to the existence of RARE sequences in the promoter regions of RAR $\alpha$ 2,  $\beta$ 2 and  $\gamma$ 2 which can be upregulated by RA (Maden et al, 1988, Giguere et al, 1994). This allows for the autoinduction of the RARs which gives the potential for amplification of the retinoid signal. The expression of RAR $\alpha$  and RAR $\gamma$  is less sensitive to RA and inducible by only high levels of the ligand, nevertheless, both RAR $\alpha$  and  $\gamma$  promoters contain similar RAREs (Lehmann et al, 1992; Leroy et al, 1991). However, the RAR $\alpha$  RARE has a lower affinity for RARs than the RAR $\beta$  RARE (Leroy et al, 1991) hence the difference in sensitivity towards RA.

The retinoid receptors can regulate transcription without binding to specific DNA sequences but by interaction with other transcription factors (Yang Yen et al, 1991; Schule et al, 1991). The retinoid receptors interact with nuclear proteins that function as repressors or coactivators. Binding of ligand causes a conformational change of the receptor thereby altering the interaction of the receptor with other nuclear proteins. In one scenario, binding of RA to RAR induces dissociation of a repressor and allows association of a coactivator resulting in transactivation of the target gene (von Bauer et al, 1996). In another scenario, binding of RA leads to binding of a repressor to the receptor resulting in the repression of transcription of the target gene (Chen and Evans, 1995). Transcriptional repression may involve the formation of inactive heterodimers which compete for DNA binding sites.



Another mechanism is the direct inhibition of gene transcription, termed silencing. RAR $\alpha$  exhibits silencer activity (Baniahmad et al, 1992) however, the repression effect is cell-type specific where in certain cells, RAR $\alpha$  may show repressor activities but may also act as a weak activator in other cell types.

#### *Unique functions of each receptor subtype and isoforms*

It had been suggested that each RAR subtype and isoform may perform unique functions due to the observation of distinct spatiotemporal expression patterns for RAR (and RXR) transcripts in the developing embryo and various adult tissue. A tissue-specific physiological role has been identified for RAR $\alpha$  in spermatogenesis (Lufkin et al, 1993) whereas RAR $\gamma$  is linked to major functions in the skin (Lohnes et al, 1993). Evidence exists to support the idea that RAR subtypes perform unique function(s) in cancers, such as some human squamous cancer cell lines (Hu et al, 1991), and in many human lung carcinoma cell lines (Gebert et al, 1991, Houle et al, 1991). In these cancerous cells, RAR $\alpha$  and  $\gamma$  are often expressed constitutively but RAR $\beta$  is suppressed. The normal counterparts of these cells express RAR $\beta$  suggesting selective suppression of RAR $\beta$  is related to the process of malignant transformation in epithelial cells. Reduced expression of RAR $\beta$  has also been associated with head and neck cancer (Xu et al, 1994). A potential role for RAR $\alpha$  and  $\gamma$  during the development and/or progression of squamous cell carcinomas (SCC) has been suggested by Xu et al, (1996) whose study of SCC showed that 94% of the samples analysed had reduced expression of RAR $\alpha$  and  $\gamma$  in comparison to normal epidermis. Inhibition of breast cancer cells by retinoids selective for RAR $\gamma$  has also been demonstrated (Fanjun et al, 1996), implicating a role for RAR $\gamma$  in the growth inhibitory effect of the retinoids.

Specific isoforms of a receptor may also express unique functions as they appear to be regulated by tissue specific expression (Zelent et al, 1991; Leroy et al, 1991). RAR $\beta$ 2 has been shown to have tumour suppressor activity in certain lung cancer cells (Berard et al, 1994) and is induced in many tissues by exogenous all-trans RA (de The et al, 1989; Lovat et al, 1993; Redfern et al, 1990; 1994; Viallet et al, 1991). In contrast, RAR $\beta$ 4 is positively correlated with neoplasia in lung and breast tissue (Houle et al, 1993; Berard et al, 1994). The RAR $\beta$ 4 isoform lacks all except 4 amino acids of the A domain (Nagpal et al, 1992) suggesting that this isoform could act as a dominant negative receptor able to bind to a RARE in a ligand-dependent manner but unable to activate transcription.

### *Null Mice Models*

The importance of the RAR *in vivo* can be shown experimentally using the null mouse model. In this system, mice deficient for either RAR $\alpha$ 1 (Li et al, 1993), RAR $\beta$ 2 (Mendelsohn et al, 1994) or RAR $\gamma$ 2 (Lohnes et al, 1993) were found to be phenotypically normal. RAR $\beta$ -null homozygotes were apparently viable and fertile (Luo et al, 1995) whereas RAR $\alpha$ -null homozygotes displayed a limited range of defects consisting of early post-natal lethality and testis generation (Lufkin et al, 1993). RAR $\gamma$ -nulls displayed growth deficiency, early lethality and male sterility due to squamous metaplasia of seminal vesicles and prostate (Lohnes et al, 1993). Many more abnormalities were observed in compound null mutants (RAR $\alpha$ 1 $\beta$ 2,  $\alpha$ 1 $\gamma$ 1 etc) which died *in utero* or soon after birth. Mice had multiple eye, skeletal, neck, trunk and abdominal region abnormalities, similar to those observed in vitamin A deficiency syndrome (VAD) (Wilson, 1953). The data from these null mice suggests the possibility of functional redundancy existing between receptors, such that loss of one isoform may be rescued by a different isoform of the same subtype, and does not rule out the possibility of the existence of some other compensatory mechanism. Little work has been carried out on RXR null mice, however, research so far has shown RXR $\alpha$  homozygous null mice suffer from embryonic lethality with cardiac defects and liver malfunction (Sucov et al, 1994). These may result as a lack of RXR to form heterodimers with RAR which would interfere with retinoid-dependent pathways.

## **1.9. Introduction to pentoxifylline (PX)**

### *PX as a Phosphodiesterase Inhibitor*

PX [3, 7-dimethyl-1 (5-oxohexyl)-xanthine], also known as oxpentifylline, is a methylxanthine derivative with properties similar to theobromine, caffeine and theophylline (Ward and Clissold, 1990).

Pentoxifylline is a specific inhibitor of cAMP-specific phosphodiesterases (Stefanovich V, 1974; Meskini et al, 1994) enhancing intracellular levels of cAMP. The increase in cAMP levels results in the activation of cAMP-dependent protein kinase A which can proceed to phosphorylate many target proteins. In mononuclear and polymorphonuclear cells, PX has been reported to induce a dose-dependent increase in cAMP expression (Bessler et al, 1986) thought to be due to this inhibition of cAMP-dependent phosphodiesterase. PX has also been shown to inhibit lymphocyte activation involving a cAMP-dependent pathway that promotes downregulation of the immune response (Rosenthal et al, 1992).

### *In Vivo and In Vitro Effects of PX*

PX was initially marketed in the USA where it was found to have a protective effect against infections such as gram negative sepsis and meningitis (Ward and Clissford, 1990) and was originally introduced as a hemorrheologic agent for the treatment of peripheral vascular disease (Muller and Lehrach, 1981; Grigoleit and Jacobi, 1977). But in contrast to other methylxanthines, it has few cardiac effects (Maxwell GM, 1975). PX is usually administered through oral or intravenous routes and greater than 90% of absorbed PX is excreted in the urine in the form of six metabolic products.

PX has also been shown to alter red blood cell (rbc) physiology although the exact mechanisms are not well understood. It is thought to improve membrane deformability by increasing the amount of membrane ATP (Porsche and Stefanovich, 1978) via the inhibition of ATPase which decreases the active intracellular transport of  $\text{Ca}^{2+}$ . PX can also alter rbc membrane protein phosphorylation patterns, increase protein kinase activity and decrease  $\text{Ca}^{2+}$ -dependent  $\text{K}^{+}$  efflux (Seidler and Swislocki, 1992). As well as inducing microvascular constriction, PX also stimulates fibrinolysis, decreases plasma fibrinogen levels, prevents rbc and platelet aggregation and increases leukocyte adhesion (Ely H, 1988; Sonkin et al,

1992; Hammerschmidt et al, 1988).

PX also affects cytokine expression resulting in an inhibition of leucocyte stimulation by  $\text{TNF}\alpha$  and IL-1, and can also suppress monocyte production of  $\text{TNF}\alpha$  (Hakim et al, 1990).

### *Use of PX in Tumour Biology*

With relation to cancer biology, Ambrus et al (1991) have reported on the ability of PX to inhibit tumour implant angiogenesis using human tumour implants in the cornea of rabbits and non-human primates. In contrast, Edward and Mackie (1991) observed PX-enhanced lung colonisation by B16 melanoma cells injected into syngeneic C57BL mice. PX-treated tumour cell GAG production was reduced by 36% and the charge density of chondroitin sulphate was diminished. Berman et al, (1990) has shown PX inhibits the amount of collagen, GAG and fibronectin production. Fibroblasts cultured in the presence of PX were shown to produce twice as much collagenase activity which may explain the decreased amounts of collagen (Berman et al, 1989). Schiano et al, (1991) showed that in vitro enhancement of cisplatin-induced cytotoxicity by PX was mediated by the inhibition of DNA repair during the S and G2 cell cycle phases. PX also enhances radiation damage in murine fibrosarcomas of mice, which Lee et al (1992) suggested was as a result of increased tumour oxygenation.

### **Hypothesis**

Since the previous results from our laboratory, using the B16 murine melanoma cell line, demonstrated the ability of RA and PX to modulate experimental metastasis and adherence of the cells to ECM components, we hypothesised that either drug may also induce changes in the properties of human melanoma cell lines in vitro which could consequently alter metastasis of the human cells in vivo.

### **1.10. Aims of the study**

The overall aim of this project was to analyse the ability of RA and PX to modulate the following steps of human melanoma cell metastasis:

- 1) melanoma cell proliferation
- 2) adhesion of the tumour cells to various ECM components
- 3) tumour cell lysis by LAK cells
- 4) expression of plasminogen activator components

The human melanoma cell lines selected for this investigation were the poorly metastatic A375 and Hs294T cells and the highly metastatic C8161 cells. All three cell lines have previously been well characterised, are widely available, and have different metastatic potentials allowing cell parameters to be correlated with ability of the tumour cells to metastasise.

#### *Retinoid and PX modulation of tumour cell proliferation and cell-ECM interactions*

This study began by analysing the ability of RA and PX to modulate tumour cell growth since numerous reports demonstrate the ability of RA to exert anti-proliferative effects on a variety of tumour cells, whilst Berman et al, (1990) reported on the inhibition of human fibroblast proliferation by PX.

A tumour cell's ability to metastasise can be correlated with adhesion to certain basement membrane and ECM components, therefore, if RA or PX were to alter tumour cell adherence, this may ultimately affect tumour cell metastasis (Terranova et al, 1982; Chung et al, 1988). Both RA and PX have the ability to alter adhesion of cells to various components of the ECM. Kato and De Luca, (1987) demonstrated a RA-induced increase in adhesion of mouse fibroblasts to laminin and collagen type IV, whereas Santos et al, (1994) reported on the RA-induced increase in melanoma cell adhesion to vitronectin. In contrast, research from our own laboratory has shown B16 murine melanoma cell adhesion to laminin and collagen type IV was reduced with RA treatment (Edward et al, 1989) as was adhesion to vascular endothelial cells and subendothelial ECM (Edward et al, 1992). PX is known to prevent the TNF- $\alpha$  induced adherence of polymorphonuclear cells to vascular endothelium (Salyer et al, 1990), and can decrease IL-6-induced neutrophil

adherence and aggregation. Gastpar et al (1974; 1977) have reported on the ability of PX to prevent adherence of tumour cells to capillary endothelial cells. Similarly, our laboratory has shown murine melanoma cell aggregation and adhesion to subendothelial ECM was increased with PX treatment which may have contributed to the observed PX-induced increase in lung tumour formation (Edward et al, 1992). In this section of the study, the ability of RA and PX to alter adhesion of the C8161 cells to fibronectin, collagens type I and type IV, laminin and basement membrane was examined with the potential of correlating any changes in adherence to modulation of the tumour cell adhesion molecule profile. The ability of the A375 cells to adhere to basement membrane matrigel was subsequently examined since adhesion of these cells to matrigel had previously been reported by Hendrix et al (1990), to be reduced on treatment with all-trans RA.

#### *Retinoid and PX modulation of tumour lysis*

The effect of either drug on tumour cell adhesive properties was preceded by an investigation into the effect of RA and PX on lysis of all three cell lines by both unactivated peripheral blood lymphocytes (PBL) and IL-2 activated lymphokine activated killer (LAK) cells. The effect of either drug treatment on mICAM-1 expression was subsequently examined followed by analysis of the role of mICAM-1 and MHC class 1 in the tumour/LAK cell interaction. It has previously been suggested that RA may render many tumour cells more susceptible to attack by cytotoxic T lymphocytes (Smith et al, 1992), and as the promoter region of the gene for ICAM-1 contains a RARE (Aoudjit et al, 1994), this suggests RA has the ability to alter ICAM-1 expression (Cilenti et al, 1995) which may ultimately modulate tumour cell lysis.

Although the intracellular signalling pathways for ICAM-1 are not well understood, various reports suggest PX could modulate ICAM-1 expression which potentially could affect tumour cell lysis. A report by Panettiere et al (1995) suggested cAMP-dependent pathways might be involved in ICAM-1 signalling which may ultimately alter ICAM-1 expression. PX may also exert its effect through NF $\kappa$ B (Biswas et al, 1994) and the promoter region of the gene for ICAM-1 contains an NF $\kappa$ B site (Degitz et al, 1991; Voraberger et al, 1991) which would allow for the potential of PX to alter ICAM-1 expression. In support of this, PX has been demonstrated to inhibit the protein kinase A-catalysed activation of NF $\kappa$ B in human T-cells and human embryo kidney cells (Biswas et al, 1994) which in turn, could

affect the expression of ICAM-1. Indeed, PX has been shown to reduce the expression of ICAM-1 by human epidermal Langerhans cells (de Fraissinett et al, 1990). With reference to MHC class 1 expression, PX has been reported to modulate MHC class 1 (Rosenthal et al, 1993). Therefore, the ability of this drug and also RA to alter expression of ICAM-1 and MHC class 1 may consequently affect the interaction of the tumour cells with LAK cells.

The effect of RA and PX on sICAM-1 expression was also investigated using a specific ELISA detection system. Expression of sICAM-1 was measured from each of the cell lines with and without PX/RA treatment to examine first of all, whether any of the cell lines secreted sICAM-1, and to demonstrate a possible correlation between alterations in mICAM-1 and sICAM-1 expression with either drug. Since tumour cells are thought to secrete sICAM-1 as a possible means to evade immunosurveillance, the ability of sICAM-1 to interfere with Hs294T and C8161 cell lysis was also examined.

#### *Retinoid and PX modulation of lung colony formation and uPA/tPA expression*

In this section, the experimental metastasis model was used to show the effect of RA and PX on tumour lung colony formation. Gastpar H (1974; 1977) had previously shown that PX decreased pulmonary metastasis which was in contrast to our own laboratory which reported on the PX-induced enhancement (Edward and MacKie, 1991) and the RA-induced reduction of lung colonisation by B16 melanoma cells injected into syngeneic C57BL mice (Edward et al, 1992).

The effect of PX and RA on expression of uPA and tPA followed, where previous research has shown that uPA can be regulated by increases in cAMP levels suggesting PX could alter uPA expression. In contrast, RA has been shown to decrease uPA levels in prostate carcinoma cells (Waghray and Webber, 1995) yet increase tPA expression in endothelial cells (Kooistra et al, 1995). PX and its metabolites have also been shown to stimulate the release of tPA (Tranquille and Emeis, 1991; Klocking et al, 1987) yet decrease levels of the tPA inhibitors;  $\alpha$ 1-antitrypsin,  $\alpha$ 2-antiplasmin,  $\alpha$ 2-macroglobulin (Di Perri et al, 1984).

### *Analysis of retinoic acid receptor expression*

The retinoid-induced alterations in studied stages of metastasis led to an interest in analysing the expression of the retinoic acid receptors in an attempt to gain a better molecular understanding of some of the data produced from previous sections. It was initially proposed to perform Northern blot analysis of the cell line RNA using probes specific for each receptor subtype. However, preliminary experiments raised doubts as to the identity of the probes, and due to time constraints, it was decided instead, to use the polymerase chain reaction to amplify specific regions of the DNA encoding RAR $\alpha$ ,  $\beta$  and  $\gamma$ , and to confirm the identity of the PCR products by Southern blotting, using probes specific for each receptor subtype.



## **Chapter 2. Materials and Methods**

## **2. Materials and Methods**

### **2.1. Materials**

#### *Cell Biology Reagents*

Eagle's minimal essential medium (MEM), Dulbecco's modified eagle medium (DMEM), RPMI-1640, phosphate buffered saline (PBS), fetal calf serum (FCS), penicillin/streptomycin, L-glutamine, trypsin and Nunclon tissue culture plastics were obtained from Life Technologies Ltd, Paisley, Scotland. All-*trans* RA, PX, bovine serum albumin (BSA), fibronectin, collagen type IV and laminin were purchased from Sigma Chemical Co, Dorset, England. Basement membrane matrigel was obtained from TCS Biologicals Ltd, Buckingham, England, whilst collagen type I was prepared from rat tendon. Ficoll-hypaque research grade was from Pharmacia Biotech, Hertfordshire, England. Chromium-51 was obtained from Amersham International plc, Buckinghamshire, England. Human recombinant interleukin-2 (h-rIL2), recombinant sICAM-1 and the sICAM-1 ELISA were from R&D Systems Europe, Abingdon, England. Sheep serum used in the confocal stainings was provided by the Scottish Antibody Production Unit (SAPU), Law Hospital, Scotland.

#### *Antibodies*

FITC-conjugated anti-human intercellular adhesion molecule-1 (ICAM-1) antibody and anti human ICAM-1 blocking antibody were from R&D Systems Europe, Abingdon, England. Non-specific mouse IgG control antibody and FITC-labelled sheep anti-mouse IgG were from the Scottish Antibody Production Unit (SAPU), Law Hospital, Scotland. Mouse anti-human HLA-ABC antibody was purchased from Serotec Ltd, Oxford, England. All antibodies used in the tPA and uPA ELISAs were kindly provided by Professor R. Leake, Division of Molecular Biology and Biochemistry, Institute of Biological and Life Sciences, Glasgow University, Scotland. The secondary FITC-conjugated sheep anti-rabbit IgG antibody used in the confocal analysis of tPA and uPA was obtained from the Scottish Antibody Production Unit (SAPU), Law Hospital, Scotland.

### *Plasminogen activator ELISA buffers*

All buffers were kindly provided by Professor Robin Leake, Division of Biochemistry and Molecular Biology, Institute of Biological Life Sciences, Glasgow University. The coating buffer contained 15mmol/l sodium carbonate, 35mmol/l sodium hydrogen carbonate at pH 9.6. The dilution buffer was prepared by adding 1% (w/v) BSA to 0.1% (v/v) in Tween-20 prepared in PBS. The washing buffer consisted of 0.1% (v/v) Tween-20 in PBS and the substrate buffer was made of 76mmol/l sodium dihydrogen phosphate and 35mmol/l citric acid, pH5.0.

### *Molecular Biology Reagents*

RNA Isolation solution (RNAzol) and oligonucleotides were purchased from Genosys, Cambridge, England. RNEasy Spin columns, Plasmid Midi-kit and taq polymerase were obtained from Qiagen, Surrey, England. Deoxynucleotide triphosphates were from Pharmacia, Herts, England and superscript II reverse transcriptase was from Gibco BRL, Paisley, Scotland. Sequenase T7 polymerase sequencing kit, Hbond nucleic acid transfer membrane and [ $\gamma$ -<sup>32</sup>P]ATP (1000-3000Ci/mmol) were both purchased from Amersham International plc, Buckinghamshire, England. XL-1 Blue bacteria (referred to in text as *E.coli*), NucTrap gel filtration columns were obtained from Stratagene, Cambridge, England and polynucleotide kinase from Promega, Southampton, England. The TA Cloning kit was from Invitrogen, The Netherlands whereas the Rapid Pure Minipreps kit was obtained from Bio101 Inc, La Jolla, California.

### *Preparation of Buffers*

RNase-free 10x MOPS buffer used in the gel electrophoresis of RNA was prepared by adding 0.1% (v/v) DEPC to 0.2M MOPS (pH7.0), 0.05M sodium acetate and 0.1M EDTA (pH8.0). The solution was left to shake overnight then autoclaved to inactivate the DEPC. TBE buffer was prepared by adding 75mM tris to 25mM boric acid and 0.1M EDTA, adjusting the pH to 8.9.

TE buffer was prepared by the addition of 10mM Tris-HCl (pH7.6) to 1mM EDTA.

For Northern transfer, 20x SSC was made by adding 3M sodium chloride to 0.3M sodium citrate, adjusted to pH 7.0. 20x SSPE used in Southern transfer was prepared by adding

3M sodium chloride and 200mM sodium dihydrogen phosphate to 20mM EDTA with the pH adjusted to 7.4. Prehybridisation solution and hybridisation solution was prepared by adding 5x SSPE, 5x Denhardt's solution (1% SDS w/v; 1% PVP w/v; 1% BSA w/v in distilled water), 100µg/ml denatured sheared salmon sperm DNA and 50% (v/v) formamide. 0.04% methylene blue and 0.5M sodium acetate (pH5.2) were used to produce the methylene blue stain.

#### *Preparation of bacterial culture solutions*

LB agar solution was prepared by adding 1% (w/v) Select peptone 140, 0.5% (w/v) yeast extract, 1.2% (w/v) agar to 0.5% (w/v) sodium chloride.

LB broth was prepared by adding 1% (w/v) Select peptone 140, 0.5% (w/v) yeast extract (w/v) to 0.55% (w/v) sodium chloride.

Both solutions were prepared in distilled water, dissolved by microwaving, and autoclaved prior to use.

## **2.2. Cell Culture**

The highly metastatic human melanoma C8161 cell line was established from an abdominal wall metastasis and characterised by Welch et al (1991) and was kindly supplied by Mary J.C. Hendrix, University of Arizona, Tucson, AZ, USA. The low metastatic Hs294T cell line (Creasey et al, 1979), derived from a lung node metastasis, and the low metastatic A375 cell lines were obtained from the American Tissue Culture Collection (ATCC, HTB-140). The MCF-7 human breast adenocarcinoma cell line, used as a positive control in the retinoic acid receptor analysis, was purchased from the European Collection of Animal Cell Cultures (ECACC). All cell lines were routinely cultured in MEM supplemented with 10% heat-inactivated FCS, penicillin (100units/ml) and streptomycin (100µg/ml). Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. Viability of the tumour cells was measured by trypan blue staining where cells which did not take up the dye were defined as viable and cells which did were described as non-viable. 0.5ml of a 0.4% (w/v) trypan blue solution was added to 0.3ml PBS and 0.2ml cell suspension and left for 5min before cells were counted using a hemocytometer.

## **2.3. Methods**

### **2.3.1. Growth Assays**

Tumour cells were set up at the appropriate densities and incubated for 4hr in complete medium before the addition of either PX (10µg/ml-500µg/ml) or RA ( $10^{-10}$  M -  $10^{-5}$  M) to fresh medium. Media changes took place on days 2 and 4. Cells were detached on days 1, 2, 3, 4 and 7 using 0.05% trypsin in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free PBS/EDTA and counted in a Coulter counter.

### **2.3.2. Adhesion Assays**

Tissue culture dishes were coated with substrate concentrations at which maximal adhesion was observed following 1hr incubation at 37°C. Excess substrate was removed and rinsed with PBS. Remaining protein absorption sites were blocked with BSA (10mg/ml) for 2hr at 37°C after which excess BSA was washed away with PBS.  $7.5 \times 10^4$  tumour cells were added to each prepared well, and after the appropriate incubation time, non-adherent cells were removed before wells were washed twice with PBS. Tumour cells were detached using trypsin/EDTA and samples removed for counting on a Coulter counter.

### **2.3.3. Assessment of peripheral blood lymphocyte and lymphokine activated killer cell-mediated tumour cell lysis**

#### *Preparation of Lymphocytes*

80ml of heparinized blood from a healthy volunteer was subjected to Ficoll density gradient centrifugation and the peripheral blood mononuclear cell layer removed. Adherent cells were removed by two cycles of adherence to tissue culture plastic. The non-adherent peripheral blood lymphocytes (PBL) were collected and maintained in RPMI supplemented with 10% FCS and lymphocytes activated by the addition of 2nM human rIL-2 to  $10^6$  cells for at least 6 days to produce lymphokine-activated killer cells (LAK).

### *Chromium-51 release assay*

$5 \times 10^3$  tumour cells, either untreated or treated with  $10^{-6}$ M RA or 250 $\mu$ g/ml PX for 4 days, were radiolabelled with Chromium-51 (400 $\mu$ Ci/ $10^7$  cells, specific activity 200-500mCi/mg) and incubated for 2hr at 37°C. Excess radiolabelled chromium was removed and labelled tumour cells incubated with varying numbers of lymphocytes to produce the effector:tumour cell ratio (E:T). After 4hr incubation, a 70 $\mu$ l sample of supernatant was removed for liquid scintillation counting (Canberra - Packard). Spontaneous release was measured by incubating the tumour cells in complete media and maximum release determined by lysing the cells in 0.1M HCl. For all the assays shown, spontaneous release was always less than 10% of the maximum release. Blocking anti-ICAM-1 antibody and blocking MHC class 1 antibodies were used at concentrations ranging from 1 $\mu$ g/ml to 25 $\mu$ g/ml, and a non-specific mouse IgG antibody used as an isotype control. sICAM-1 was added at a concentration of 100ng/ml.

### *Quantitative analysis of cytotoxicity*

The percentage of lysis occurring was calculated as follows :

% specific lysis =  $(\text{dpm experimental well} - \text{dpm spontaneous release}) \div (\text{dpm maximal release} - \text{dpm spontaneous release})$ .

### *Immunofluorescence analysis of mICAM-1 and MHC Class 1*

For the ICAM-1 staining, tumour cells were fixed using acetone and labelled with 200 $\mu$ l of FITC-conjugated mouse anti-human ICAM-1 antibody (20 $\mu$ g/ml) for 2hr at room temperature in the dark. Excess antibody was removed by washing cells four times in PBS to remove unbound antibody. Propidium iodide (1 $\mu$ g/ml) was used as a counterstain prior to examination using a confocal microscope.

For the MHC class-1 staining, tumour cells were incubated with 10% sheep serum before the addition of 200 $\mu$ l of mouse anti-human HLA-ABC antibody for 2hr at room temperature. Excess antibody was removed by washing cells three times in PBS. Secondary FITC-labelled sheep anti-mouse IgG was added at a 1:200 dilution and left for 2hr at room temperature in the dark. Excess antibody was removed by washing cells four times in PBS to remove unbound antibody. Propidium iodide (1 $\mu$ g/ml) was used as a counterstain prior to examination using a confocal microscope.

### *Flow Cytometric Analysis of mICAM-1*

Tumour cells were prepared for fluorescent-activated cell sorter analysis by the addition of 10µl of FITC-conjugated ICAM-1 antibody (equivalent to one test as described by R&D systems) to  $10^6$  tumour cells and incubated for 15min. Excess antibody was removed by washing the cells twice in PBS. Fluorescent staining was analysed using a FACscan (Becton Dickinson). The corresponding background fluorescence was obtained using tumour cells minus antibody. Intensity of fluorescence was recorded on a logarithmic scale.

### **2.3.4. Soluble intercellular adhesion molecule-1 assay**

The procedure for measuring sICAM-1 was as described in the R&D Systems parameter handbook. Briefly, 100µl of anti-ICAM-1 horse radish peroxidase conjugate was added to each well coated with mouse anti-human ICAM-1 antibody. This was followed by the addition of 100µl of diluted supernatant from cells pretreated with either 10µg/ml - 250µg/ml PX or  $10^{-10}$  M -  $10^{-5}$  M RA for 4 days. The plate was incubated for 2hr at room temperature before the contents of each well was removed. Wells were washed 6 times with 300µl of wash buffer, after which 100µl of substrate was added to each well and left to incubate at room temperature for 30min. 100µl of the stop solution was added to each well and the optical density of the plate read in a microtitre plate reader set at 450nm with correction of 620nm.

### **2.3.5. Tumour Lung Colonisation**

Tumour cells were set up at the appropriate densities and pretreated for 4 days with either  $10^{-6}$  M RA or 250µg/ml PX. Cells were then trypsinised and counted on a Coulter counter. Treated or untreated tumour cells were injected into the tail vein of athymic nude mice (nu/nu), with 8 mice per experimental group. Mice were killed after 21 days, the lungs excised and nodules counted.

### **2.3.6. Plasminogen activation analysis**

#### *Confocal Microscopy of Membrane-Associated uPA and tPA Expression*

Tumour cells were fixed using acetone and incubated with 10% sheep serum before being labelled with 200µl of rabbit anti-human tPA or uPA antibody (20µg/ml) for 2hr at room temperature. Excess antibody was removed by washing three times in PBS. FITC-labelled secondary sheep anti-rabbit IgG was added at 1:200 dilution and left for 2hr at room temperature. Cells were washed four times in PBS to remove unbound antibody. Propidium iodide (1µg/ml) was used as a counterstain prior to examination using a confocal microscope.

#### *ELISA measurements of secreted uPA and tPA*

ELISAs were performed essentially as described by Grebenschikov et al, 1997. Briefly, 96 well microtitre plates were coated with sheep anti-chicken IgG and incubated at 4°C overnight. Plates were washed 4 times in washing buffer before the addition of 300µl of blocking buffer to each well. Plates were incubated at 37°C for 2hr then washed four times in washing buffer. 100µl of controls and samples (of various dilutions) were transferred into each well and incubated at 4°C overnight. After washing samples 4 times with washing buffer, 100µl of the appropriate catching antibody (chicken anti-human uPA/tPA) was added to each well and plates incubated at 37°C for 2hr. Plates were washed a further 4 times before the addition of the appropriate tagging antibody (rabbit anti-human uPA/tPA) to each well and plates incubated at room temperature for 2hr. Plates were washed 4 times then incubated for 2hr at room temperature with detecting antibody (goat anti-rabbit IgG). Samples were washed a further 4 times then 100µl substrate solution added to each well. Plates were incubated in the dark for 30min at room temperature. The reactions were stopped with 100µl of 1M sulphuric acid and readings taken in a spectrophotometer at wavelengths 492nm/620nm.

### **2.3.7. Statistical analysis of data**

Unless otherwise stated in the figure legend, samples were performed in triplicate and expressed as the average  $\pm$  standard error of the mean (SEM). Statistical analysis of the data was performed using the students 2-tailed T-test.



### **2.3.8. Analysis of RAR expression**

#### *Preparation of Competent E.coli*

Competent *E.coli* were prepared by inoculating 2ml of LB broth with a single colony of XL-1 Blue bacteria which were incubated overnight at 37°C. 1ml of this culture was added to 100ml LB broth and cells allowed to grow with vigorous shaking at 37°C until the density reached  $5 \times 10^7$  cells/ml (ie. where the optical density at 550nm equalled 0.2). Cultures were centrifuged and cells resuspended in 50ml ice-cold 50mM CaCl<sub>2</sub> containing 10mM Tris-HCl (pH 8.0). The cells were pelleted and resuspended in 6ml ice cold 50mM CaCl<sub>2</sub> and stored on ice for immediate use.

#### *Transformation of the RAR containing pSG5 Vectors*

The pSG5 plasmids containing either the cDNA insert for  $\alpha$ ,  $\beta$  or  $\gamma$  RAR were kindly supplied by P. Chambon (INSERM, Strasbourg, France) as shown in Figure 45, Chapter 3. On arrival, plasmids were eluted from Whatman 5MM paper with 50 $\mu$ l 10mM Tris-HCl, pH 7.6. The samples were vortexed and centrifuged, and the supernatant used to transform bacteria. The vectors were transformed into competent *E.coli* by adding 5 $\mu$ l of vector to 0.2ml cells with 2 $\mu$ l of 0.5M  $\beta$ -mercaptoethanol and left for 30min on ice. Cells were subject to heat shock at 42°C for 30sec before the addition of 450 $\mu$ l SOC medium. Cells were incubated for 1hr at 37°C then 50 $\mu$ l and 200 $\mu$ l aliquots were plated on agar plates containing ampicillin (100 $\mu$ g/ml). Plates were inverted and left overnight at 37°C in a incubator and plated on LB agar containing ampicillin (100 $\mu$ g/ml). The following day, 3 colonies of each receptor were picked and cultured overnight in 3mls LB broth containing ampicillin (100 $\mu$ g/ml). The resultant suspension was separated into 2 tubes and subject to centrifugation. One sample was added to 15% glycerol in LB broth and stored at -70°C and the other sample was used to purify vector using Rapid Pure Minipreps kit. The eluted plasmid DNA was subject to the following restriction enzyme digests for 2hr, 37°C using the following enzymes:

RAR $\alpha$  - EcoR1, Pst1, EcoR1/Pst; RAR $\beta$  - EcoR1; RAR $\gamma$  - EcoR1, Pst1, EcoR1/Pst1.

10 $\mu$ l of each digest was loaded on a 1% agarose/1x TBE gel containing ethidium bromide (1 $\mu$ g/ml) and run at 60V for 60-90min. The gel was visualised by UV transillumination and photographed.

### *Amplification of pGS5 Vector Inserts*

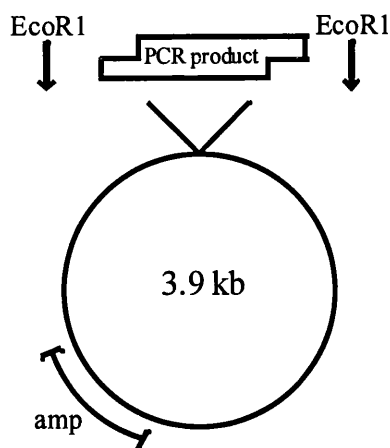
Since the restriction enzyme digests produced unexpected results, it was decided to try to amplify specific regions of the RAR $\alpha$ ,  $\beta$  and  $\gamma$  inserts and attempt to subclone them into the pCR2.1 vector for partial sequence analysis.

The following set of primers obtained from Genosys, Cambridge were used to amplify the inserts of human nucleotide sequences contained within the purified vectors:

		Amplicon (bp)
RAR $\alpha$	5' TGGGTGGACTCTCCCCGCCA 3' 5' ACCTCCGGCGTCAGCGTGTA 3'	486
RAR $\beta$	5' CACTGGCTTGACCATCGCAGACC 3' 5' TTGAATGAGAGGTGGCATTGATCCA 3'	522
RAR $\gamma$	5' GGCCTGGGCCAGCCTGACCTC 3' 5' AGAGCCCCAGATCCAGCTGCAC 3'	641

The vectors were used directly as templates for polymerase chain reaction and used as serial 10-fold dilutions in sterile water. 15 $\mu$ l cDNA template was added to a mix containing 1x PCR buffer (Qiagen), 1x Solution Q (Qiagen), 10mM each of dATP, dCTP, dGTP and dTTP, 1 $\mu$ mol of each primer and 2.5 units taq polymerase made up to a final volume of 50 $\mu$ l. Amplification began with denaturing the cDNA for 5 min at 95°C followed by 90s at 55°C for annealing and 90s at 72°C for extension. The next cycle began with 90s at 95°C and was repeated for a total of 35 cycles. Reactions with no vector added were used as negative controls, and each reaction was performed in 100 $\mu$ l overlaid with mineral oil. Amplification took place using a CrocodileIII, TC2000 PCR thermal cycler (Appligen, Oncor, USA).

*Subcloning of the PCR product into the pCR2.1 vector (TA vector)*



**Figure 3.** The pCR2.1 cloning vector.

Ligation of each PCR product into the pCR2.1 cloning vector (also known as the TA vector) (Figure 3) was performed using the TA cloning kit. Ligation reactions were incubated at 14°C for 4hr. Competent *E.coli* were prepared and 2µl of ligation mixture transformed into cells as already described. 50µl and 200µl aliquots were plated and 10 colonies of each receptor picked and cultured overnight at 37°C in 3ml LB broth containing ampicillin (100µg/ml). The vectors from each culture were purified using the Plasmid Midi Kit. Purified vector was subject to EcoRI digestion and a sample was electrophoresed through a 1% agarose/1x TBE gel containing ethidium bromide to confirm the presence of the insert.

*Sequencing of the pCR2.1 vector containing PCR insert*

5µg of purified vector containing the correct insert was denatured in 0.2M NaOH followed by neutralisation in 0.45M sodium acetate (pH5.4) and precipitation in 3 volumes of absolute ethanol. Samples were stored at -70°C for 30min then washed in 70% ethanol. Samples were heated at 60°C to evaporate residual ethanol. Sequencing was carried out using the Sequenase T7 polymerase Sequencing Kit using the supplied M13 forward primer. The T7 primer was used to sequence the pSG5 RARγ vector.

M13 forward primer    5' GTAAAACGACGGCCAG 3'  
T7 primer                    5' TAATACGACTCACTA 3'

Samples were run on a 6% acrylamide/7M urea preheated gel in 1x TBE which was transferred to 3MM Watmann filter paper and dried. The gel was then exposed to film overnight.

#### *RAR $\alpha$ and $\beta$ probe production*

The RAR $\alpha$  and  $\beta$  vectors derived from the same clones as used in the sequencing reactions were used to produce probes for the Northern blot analysis. Each clone was cultured in 100ml LB broth containing ampicillin (100 $\mu$ g/ml) overnight at 37°C, the vectors were purified and subject to large scale EcoR1 digestion.

The products produced on the large scale EcoR1 digestion of RAR $\alpha$  and  $\beta$  were excised from the gel and purified using the gel extraction kit. A small aliquot from each extraction was run on a 1% agarose gel/1x TBE and visualised under UV light.

DNA probes were prepared using either a) the PCR product or b) the actual pCR2.1 vector/insert, where the insert was separated using EcoR1 restriction enzyme digestion (as previously described) and purified from the gel using Quiagen Quick Spin columns.

5' End-labelling of both the PCR products and the purified digests was performed using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (1000-3000Ci/mmol) in a final volume of 70 $\mu$ l. For random labelling, the Klenow fragment of DNA polymerase and [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) was used. Probes were passed through a NucTrap gel filtration column to remove unincorporated nucleotide, denatured in 1M NaOH and incubated at 37°C for 10min.

#### *Preparation of RNA for Northern Blot Analysis*

Cells were detached from tissue culture plates using trypsin/EDTA and resuspended in MEM containing 10% FCS. Cells were subject to centrifugation and the pellet lysed in RNA Isolation solution, (0.2ml/10<sup>6</sup> cells). Chloroform was added to each sample (0.2ml/2ml sample) and the organic and aqueous phases separated by centrifugation at 12000g for 15min, 4°C. The aqueous phase was removed and precipitated with an equal volume of isopropanol. Samples were left for 15min at 4°C before being centrifuged for a further

15min. The RNA pellet was washed once with 75% ethanol then resuspended in RNase-free water. Quantification of the RNA sample was performed by measuring the absorbance at 260/280nm on a spectrophotometer. Samples were further purified using an RNEasy Mini Kit where necessary, based on these absorbance readings, and requantified. Samples were loaded onto a 1% agarose/1x TBE gel and stained using ethidium bromide. The gels were visualised by UV transillumination and photographed. The rest of the samples were stored at -70°C.

#### *Northern Blot analysis of tumour RNA*

10µg total RNA was precipitated in 0.1 volume of 3M sodium acetate, pH5.4 and 3 volumes absolute ethanol. Samples were stored at -70°C for 30min, centrifuged and the pellet washed in 75% ethanol and resuspended in TE buffer. Each RNA sample was electrophoresed through a 1% agarose/formaldehyde gel overnight at 30V, 4°C then transferred to a nylon membrane by capillary Northern blotting technique. The membrane was then washed in 5% (v/v) acetic acid followed by staining for 15min in 0.04% (w/v) methylene blue/5% (v/v) acetic acid to visualise RNA nucleic acids. Excess stain was removed by washing with distilled water. After removal of excess water, the RNA was cross-linked to the membrane by UV illumination.

Filters were prehybridised for 4 hr at 42°C in prehybridisation buffer. Hybridisation with the labelled probe was performed in hybridisation buffer overnight at 42°C. The blot was then washed for 2 x 15min at room temperature in the same solution followed by 2 x 15min washes in 0.1X SSC, 0.1% (w/v) SDS at 37°C then autoradiographed.

#### *Amplification of RAR $\alpha$ , $\beta$ and $\gamma$ from cell line cDNA*

5µg RNA was added to a mix containing 1µg oligo d(T)<sub>12-18</sub>, 0.02M DTT, 1mM dNTPs (Pharmacia, St Albans, Herts) and 200 units Superscript II reverse transcriptase (Gibco BRL), in a final volume of 20µl. Samples were incubated for 1hr at 42°C.

### *RAR amplification primers and conditions*

Essentially, as previously described by Harant et al, (1993), the following set of primers were used to amplify the reverse transcribed templates from Hs294T and C8161 RNA.

			Amplicons
RAR $\alpha$	sense	5' GACTGTGGAGTTCGCCAAGC 3'	620bp
	antisense	5' CCCC GCCAGGCAGCTGTAG 3'	
RAR $\beta$	sense	5' AGGAGACTTCGAAGCAAG 3'	771bp
	antisense	5' GTCAAGGGTTCATGTCCT 3'	
RAR $\gamma$	sense	5' GGAAGAAGGGTCACCTGA 3'	587bp
	antisense	5' CGGCGCCGGGCGTACAGC 3'	
GAPDH	sense	5' AGGTCGGAGTCAACGGATTTG 3'	321bp
	antisense	5' ATGGAGAAGGCTGGGGCTCAT 3'	

Amplification was achieved under standard conditions in a CrocodileIII, TC2000 PCR thermal cycler (Appligen, Oncor, USA). The reaction was performed in 50 $\mu$ l using taq polymerase, as described for the previous PCR amplifications. The amplification conditions began with denaturing the cDNA for 5 min at 95°C followed by 60s at 65°C for annealing and 60s at 72°C for extension for RAR $\alpha$  and 60s at 60°C for annealing and 60s at 72°C for extension of RAR $\beta$  and  $\gamma$ . The next cycle began with 30s at 95°C and was repeated for a total of 30 cycles. The negative controls included were a) the reverse transcription performed with no template RNA, b) the PCR mix with no template, and c) PCR performed on template RNA. The human breast carcinoma MCF-7 cell line was chosen as a positive control for RAR $\alpha$  as Widshwendter et al, (1995) had previously shown expression of RAR $\alpha$  in this cell line. This positive control was necessary as the initial run of PCR on the C8161 cells was negative even though cDNA synthesis was confirmed by amplification of GAPDH.

### *Southern Blot Analysis of PCR products.*

PCR products were subjected to electrophoresis in a 1% agarose/1x TBE gel then transferred to a nylon membrane by capillary action. The membrane was then washed in 5% (v/v) acetic acid followed by staining for 15min in 0.04% (w/v) methylene blue/5%(v/v) acetic

acid to visualise nucleic acids. Excess stain was removed by washing with distilled water, after which, the DNA was cross-linked to the membrane by UV irradiation. Synthetic oligonucleotide probes complementary to exon / exon boundary sequences were designed to confirm the identity of the PCR products, and to show that the amplification was specifically from processed mRNA.

RAR $\alpha$ probe	5' CATCCTGATCCTGCGGATCTGCACGCGGTA 3'
RAR $\beta$ probe	5' ATACACCACGGTAAGATCCTAGAATTCCAG 3'
RAR $\gamma$ probe	5' TGCCTAGATATCCTGATGCTGCGTATCTGCA 3'

5' End-labelling of the probes was performed using polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (1000-3000Ci/mmol) in a final volume of 70 $\mu$ l at 37°C for 30min. Probes were passed through a NucTrap gel filtration column to remove unincorporated nucleotide. Membranes were prehybridised for 4hr at 42°C in prehybridisation buffer. Hybridisation with the labelled probe was performed overnight at 42°C using hybridisation buffer. The membranes were then washed for 2 x 15min at room temperature in 5x SSPE, 0.1% SDS (w/v) in the same solution followed by 2 x 15min washes in 0.1x SSPE, 0.1% SDS (w/v) at 37°C then subjected to autoradiography.

## **Chapter 3. Results**

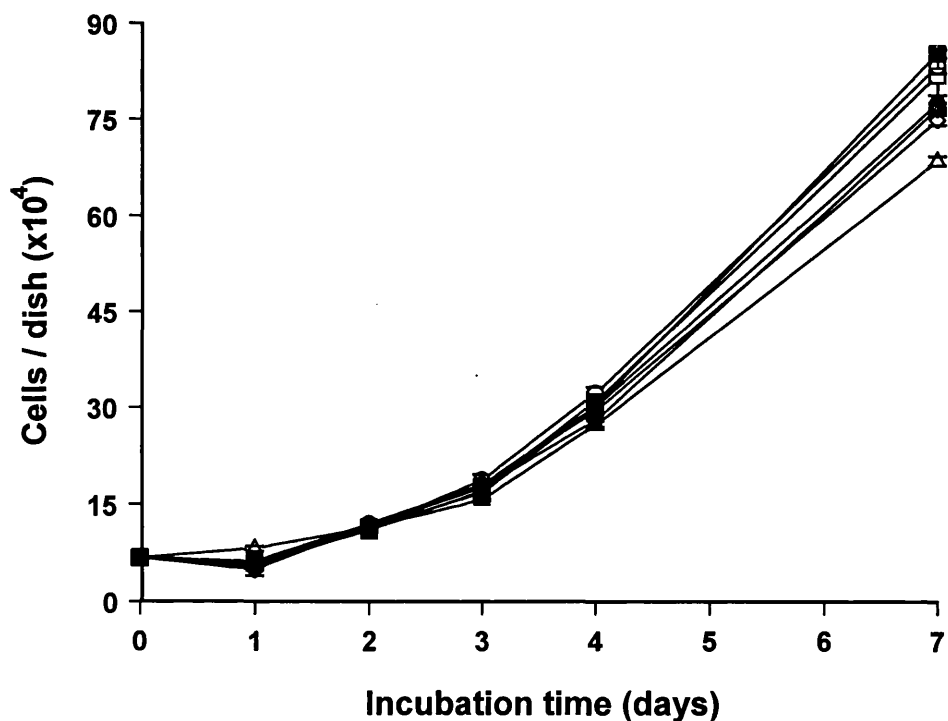


### 3. Results

#### 3.1. Retinoid-induced modulation of tumour cell growth and adhesion to various ECM components

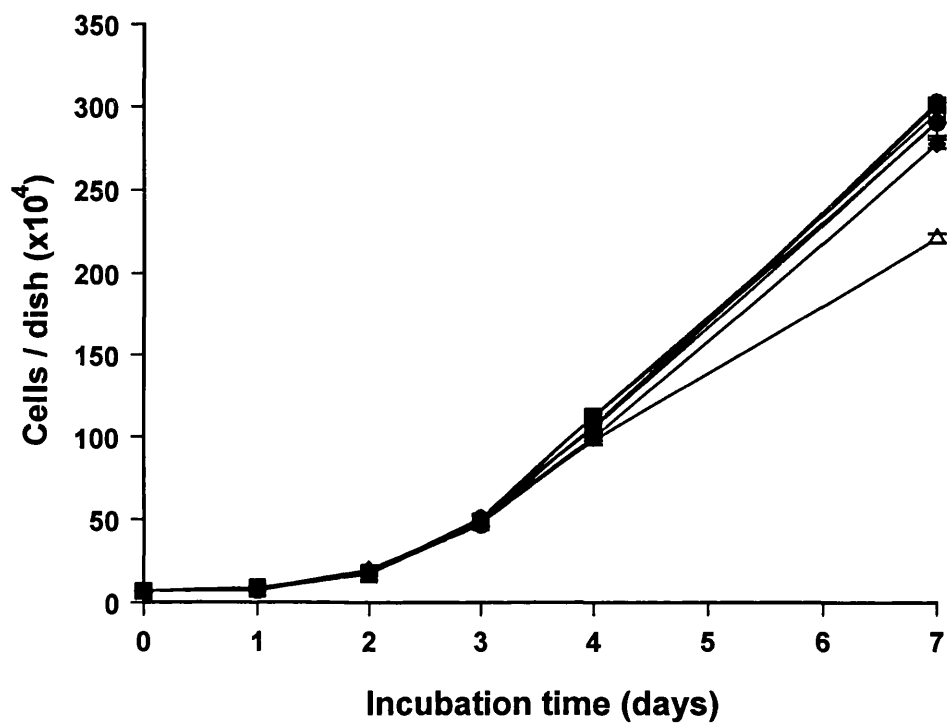
##### *Effect of RA ( $10^{-10}M$ - $10^{-5}M$ ) on tumour cell growth*

RA over a range of concentrations ( $10^{-10}M$  -  $10^{-5}M$ ) did not significantly alter Hs294T cell numbers over a four day period (Figure 4A). Only after 7 days pretreatment of the cells with  $10^{-5}M$  RA was a slight decrease in cell numbers observed from  $8.2 \times 10^5$  cells to  $6.9 \times 10^5$  cells, however, this treatment reduced viability of the cells to below 75%.



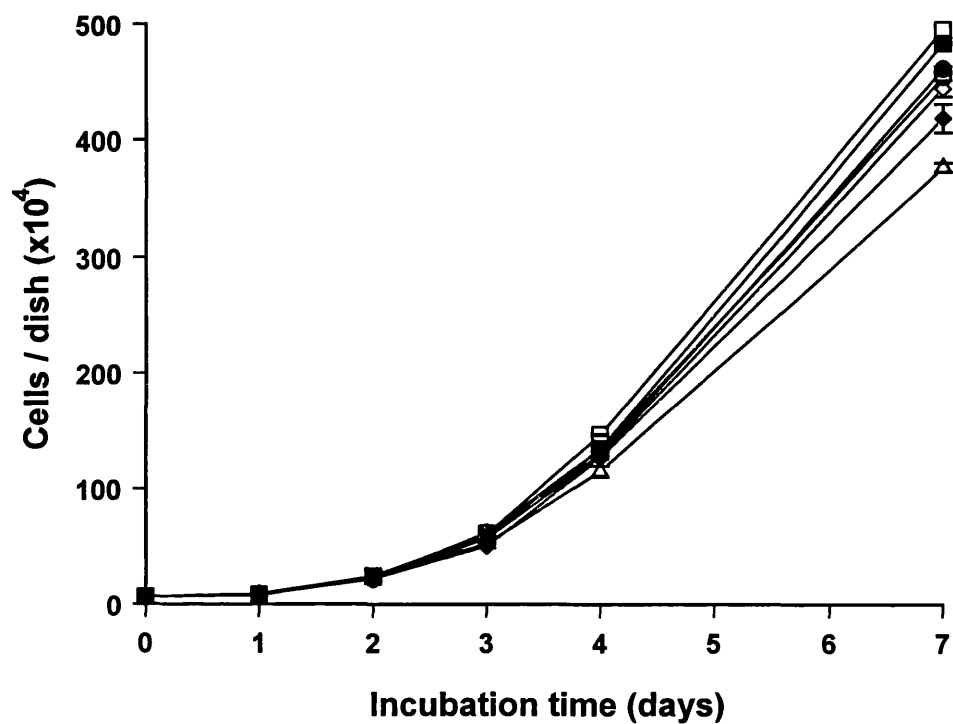
**Figure 4A.** Effect of RA (□ control; ■  $10^{-10}M$ ; ○  $10^{-9}M$ ; ●  $10^{-8}M$ ; ◇  $10^{-7}M$ ; ◆  $10^{-6}M$ ; △  $10^{-5}M$ ) on Hs294T cell numbers. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

A similar outcome was obtained using the C8161 cell line, where RA over a range of concentrations ( $10^{-10}\text{M}$  -  $10^{-5}\text{M}$ ) did not significantly alter cell numbers over a four day period (Figure 4B). After 7 days pretreatment of the cells with  $10^{-5}\text{M}$  RA, a slight decrease in cell numbers was observed from  $2.96 \times 10^6$  cells to  $2.21 \times 10^6$  cells. But again, this treatment reduced viability of the cells to below 75%.



**Figure 4B.** Effect of RA ( $\square$  control;  $\blacksquare$   $10^{-10}\text{M}$ ;  $\circ$   $10^{-9}\text{M}$ ;  $\bullet$   $10^{-8}\text{M}$ ;  $\blacklozenge$   $10^{-7}\text{M}$ ;  $\blacklozenge$   $10^{-6}\text{M}$ ;  $\triangle$   $10^{-5}\text{M}$ ) on C8161 cell numbers. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

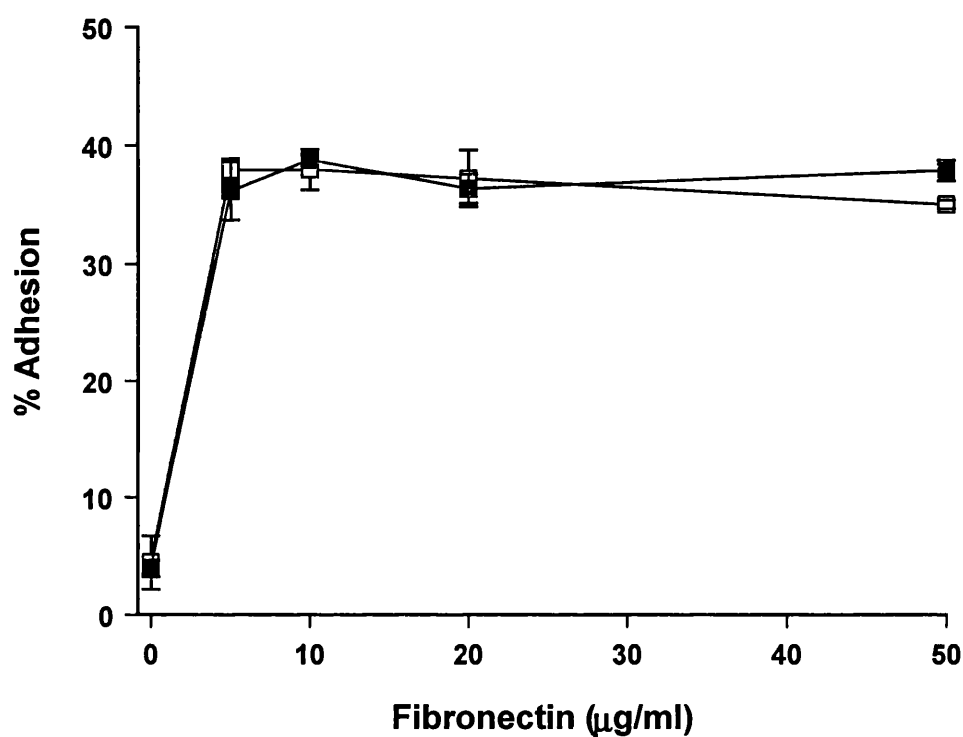
In the A375 cell line, 4 days pretreatment using a range of concentrations of RA ( $10^{-10}\text{M}$  -  $10^{-5}\text{M}$ ) did not significantly alter cell numbers (Figure 4C). The exception was using  $10^{-6}\text{M}$  -  $10^{-5}\text{M}$  RA pretreatment for 7 days. But as with the C8161 and Hs294T cells, prolonged treatment of the tumour cells at the higher concentrations of RA reduced viability to less than 75%.



**Figure 4C.** Effect of RA (□ control; ■  $10^{-10}\text{M}$ ; ○  $10^{-9}\text{M}$ ; ●  $10^{-8}\text{M}$ ; ◇  $10^{-7}\text{M}$ ; ◆  $10^{-6}\text{M}$ ; △  $10^{-5}\text{M}$ ) on A375 cell numbers. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

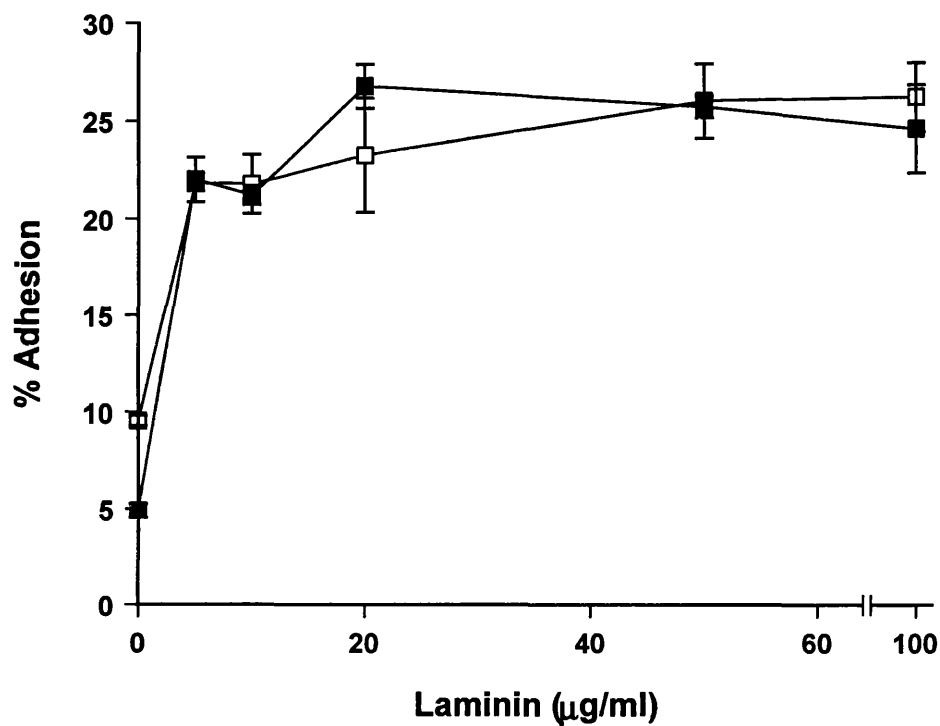
*Determining optimum concentrations of ECM components for the time-point adhesion assays of C8161 cells pretreated for 4 days with 10<sup>-6</sup>M RA.*

38% of control cells and 39% of RA-treated cells adhered to 10µg/ml fibronectin (Figure 5A). Values were similar using 50µg/ml fibronectin with 35% and 38% of control and RA-treated tumour cells adhered respectively. Therefore, the optimum concentration of 10µg/ml fibronectin was used to coat dishes in the time-point adhesion assay.



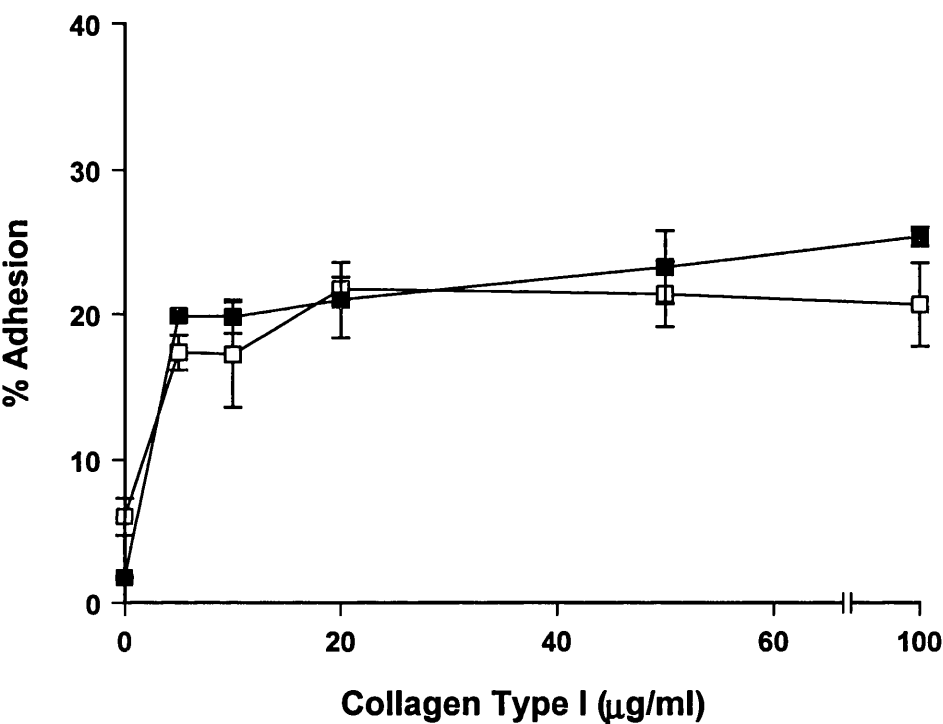
**Figure 5A.** Adhesion of C8161 cells pretreated without (□) and with 10<sup>-6</sup>M RA (■) to various concentrations of fibronectin. Values are represented as the average ± SEM of triplicate experimental dishes.

An average of 23% of control cells and 27% of RA-treated cells adhered to 20µg/ml laminin, compared to 26% adherence for both control and RA-treated cells using 50µg/ml laminin (Figure 5B). Therefore 20µg/ml laminin was selected to coat dishes in the time-point adhesion assays.



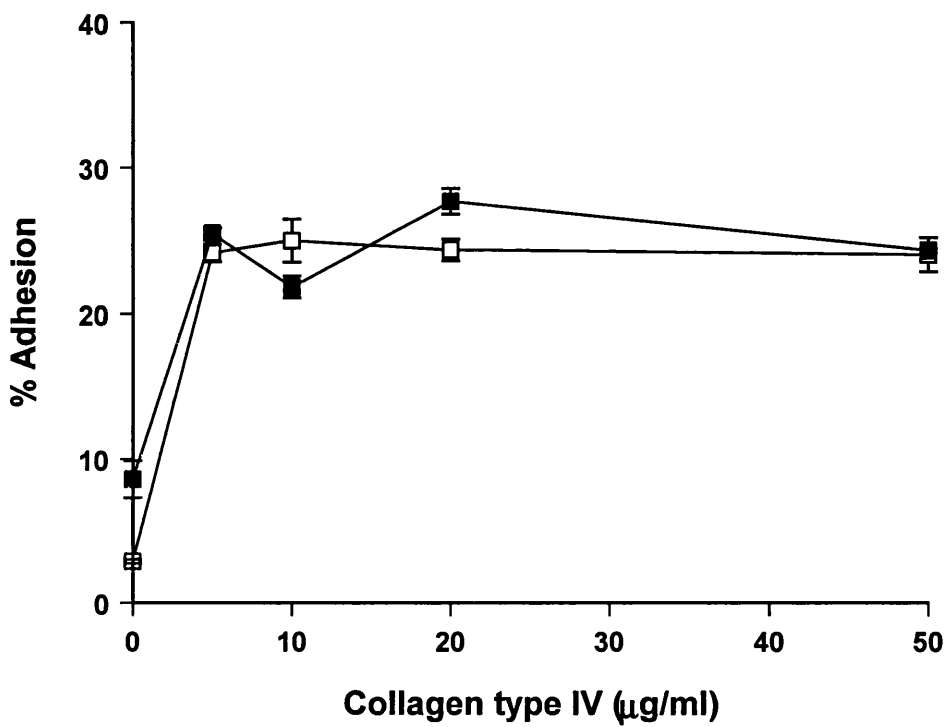
**Figure 5B.** Adhesion of C8161 cells pretreated without (□) and with 10<sup>-6</sup>M RA (■) to various concentrations of laminin. Values are represented as the average ± SEM of triplicate experimental dishes.

17% of control cells and 20% of RA-treated cells adhered to 10µg/ml collagen type 1 (Figure 5C). In comparison, using 20µg/ml substrate, 22% of control cells and 21% of RA-treated cells adhered. Values were similar at the higher concentration of 50µg/ml collagen type 1, with 21% of control cells and 23% of RA-treated cells adhering. Hence the optimum concentration of collagen type 1 used in the time-point adhesion assays was 10µg/ml.



**Figure 5C.** Adhesion of C8161 cells pretreated without (□) and with 10<sup>-6</sup>M RA (■) to various concentrations of collagen type I. Values are represented as the average ± SEM of triplicate experimental dishes.

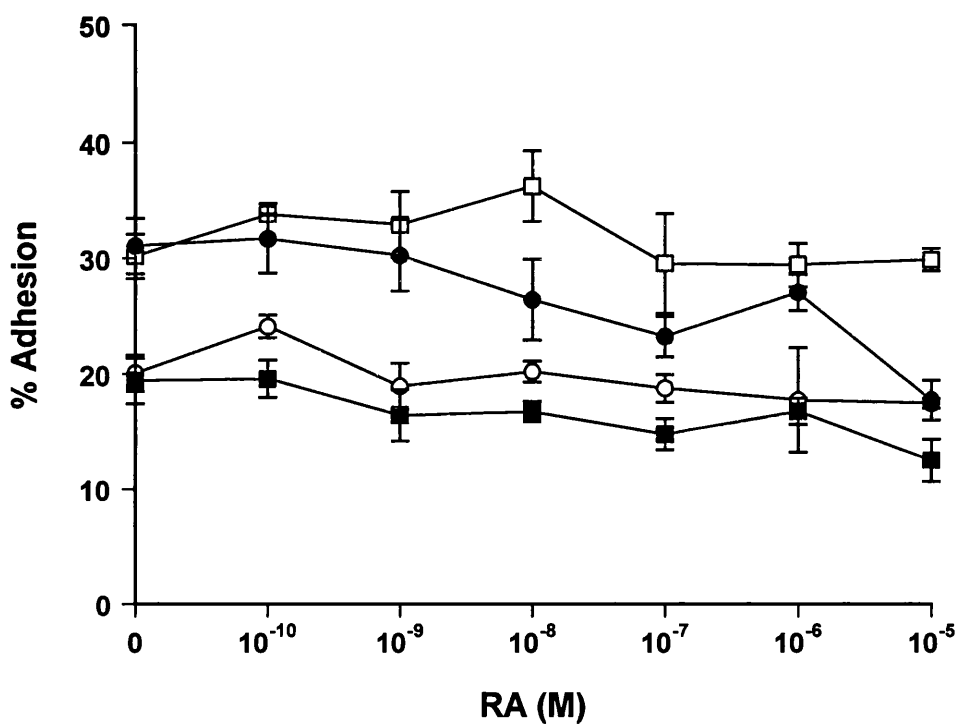
Optimum adhesion of cells to collagen type IV occurred using 5µg/ml of substrate where adhesion of both control and RA-treated cells was 24% (Figure 5D). Similar values were obtained using 10µg/ml where 25% of control cells adhered compared to 22% of RA-treated cells.



**Figure 5D.** Adhesion of C8161 cells without (□) and with (■) 4 days pretreatment of 10<sup>-6</sup>M RA to a range of concentrations of collagen type IV. Values are represented as the average ± SEM of triplicate experimental dishes.

*Effect of RA ( $10^{-10}$ M -  $10^{-5}$ M) on adherence of the C8161 cells to ECM components*

The adherence of C8161 cells to various components of the ECM did not alter significantly using a range of concentrations of RA ( $10^{-10}$ M -  $10^{-6}$ M) (Figure 6). Only at the higher concentration of  $10^{-5}$ M was there a reduction in adherence to collagen type I and IV, however, viability of the tumour cells was reduced to below 75%. Therefore, tumour cells were treated with  $10^{-6}$ M RA for use in the time-point adhesion assays.

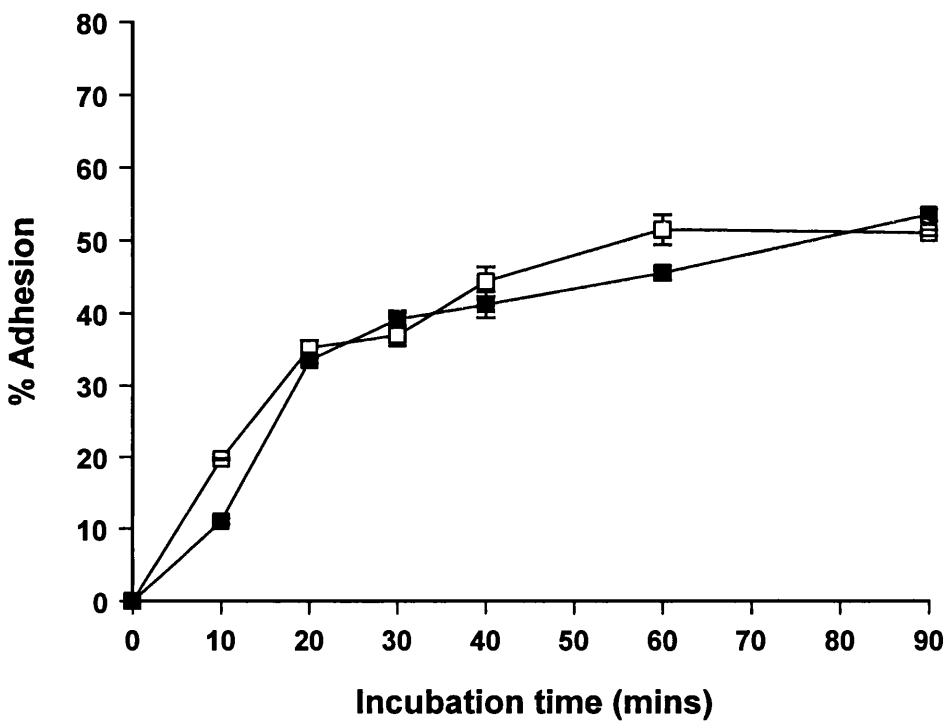


**Figure 6.** Adherence of C8161 cells pretreated with RA ( $10^{-10}$ M -  $10^{-5}$ M) to fibronectin (□); collagen type IV (■); laminin (○) and collagen type I (●). Values represent the average  $\pm$  SEM of triplicate experimental dishes where the value at zero concentration was determined by adhesion of untreated cells to substrate.



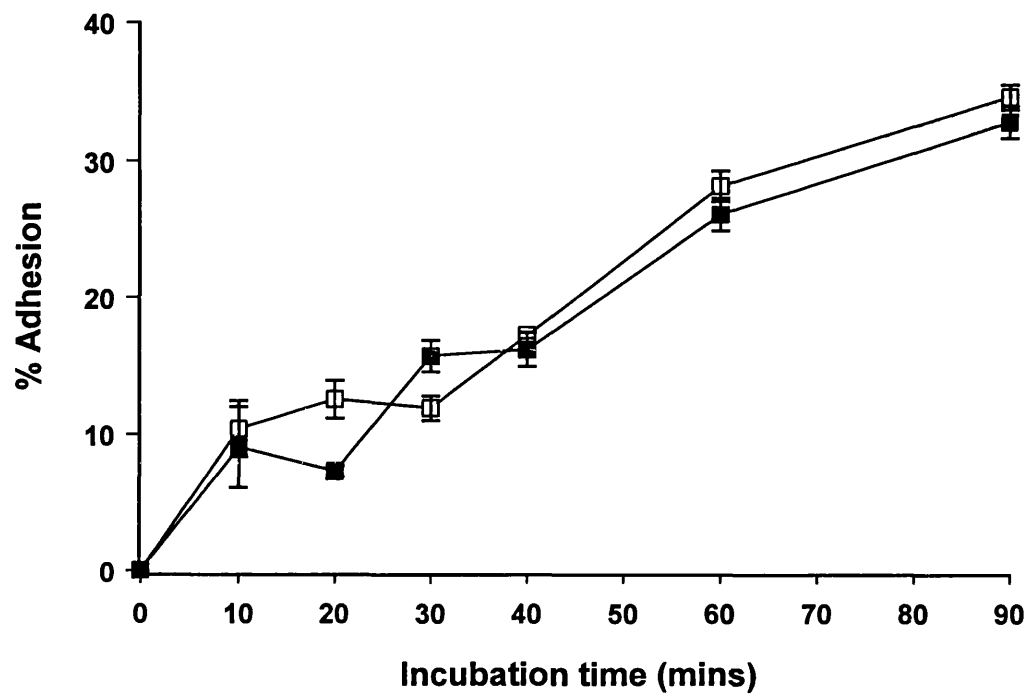
*Time point adhesion assays analysing adherence of C8161 cells with and without  $10^{-6}M$  RA pretreatment to various ECM components*

Adhesion of C8161 cells pretreated with  $10^{-6}M$  RA for 4 days to fibronectin did not differ significantly from the untreated C8161 cells of which 19% adhered to fibronectin by 10min, increasing to 37% by 30min, then to 51% by 90min (Figure 7A).



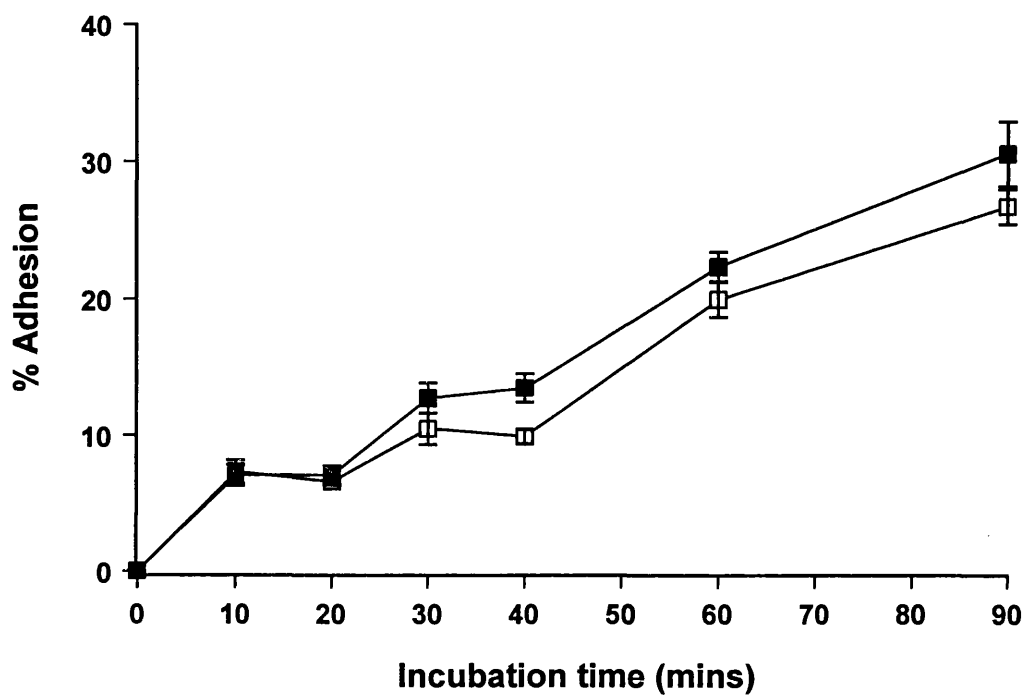
**Figure 7A.** Adhesion of untreated C8161 cells (□) and  $10^{-6}M$  RA-pretreated C8161 cells (■) to fibronectin. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

Adherence of C8161 cells to laminin was not significantly altered on pretreatment of the tumour cells with  $10^{-6}$ M RA (Figure 7B). 10% of untreated tumour cells adhered by 10min, increasing marginally to 12% by 30min and reaching 35% by 90min.



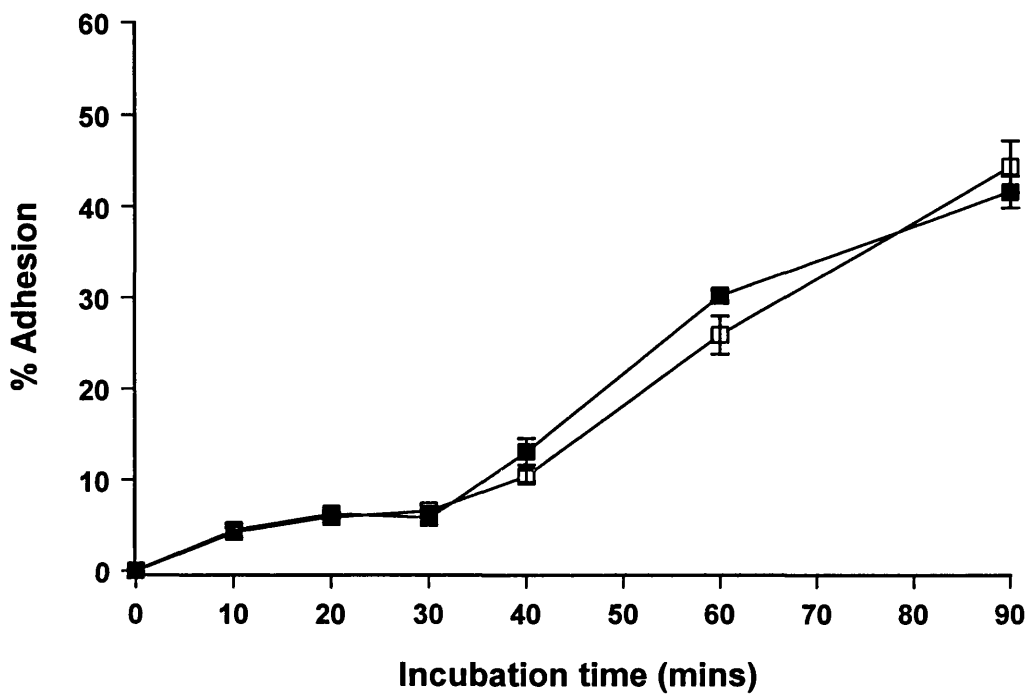
**Figure 7B.** Adhesion of untreated C8161 cells ( $\square$ ) and  $10^{-6}$ M RA-pretreated C8161 cells ( $\blacksquare$ ) to laminin. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

Adherence of C8161 cells to collagen type I was not significantly altered on pretreatment of the tumour cells with  $10^{-6}$ M RA (Figure 7C). 7% of untreated tumour cells adhered by 10min, increasing marginally to 9% by 30min, and increasing 3-fold to 27% by 90min. Values were similar in RA-treated cells.



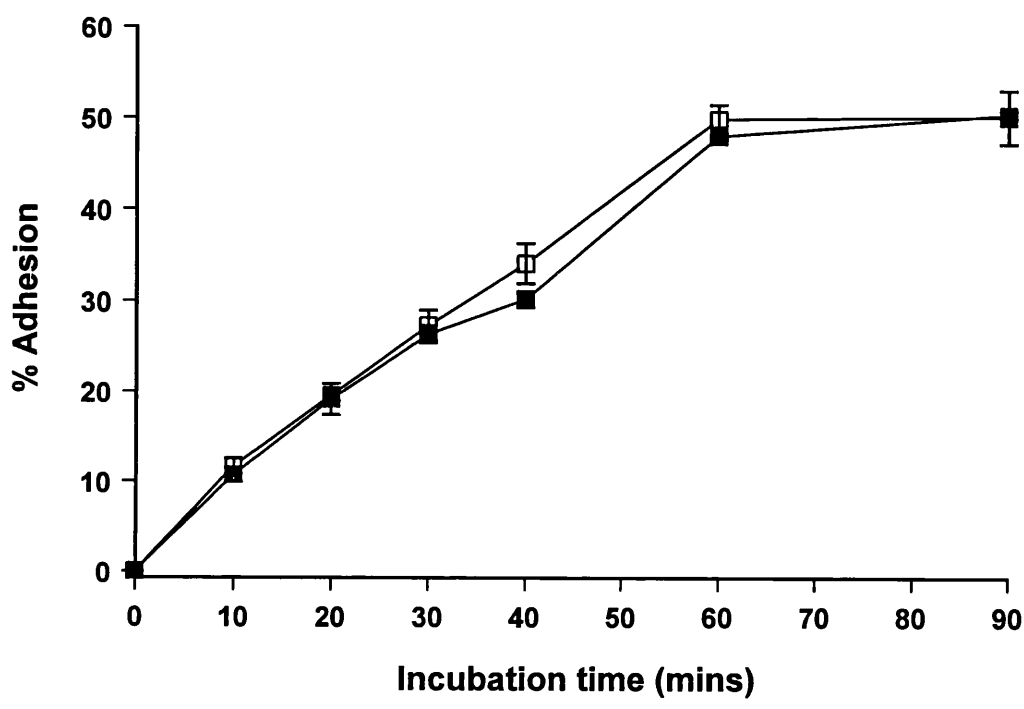
**Figure 7C.** Adhesion of untreated C8161 cells (□) and  $10^{-6}$ M RA-pretreated C8161 cells (■) to collagen type I. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

RA ( $10^{-6}$ M) did not significantly alter adhesion of the C8161 cells to collagen type IV. 4% of untreated C8161 cells adhered by 10min, compared to 7% by 30min and 44% by 90min (Figure 7D).



**Figure 7D.** Adhesion of untreated C8161 cells (□) and  $10^{-6}$ M RA-pretreated C8161 cells (■) to collagen type IV. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

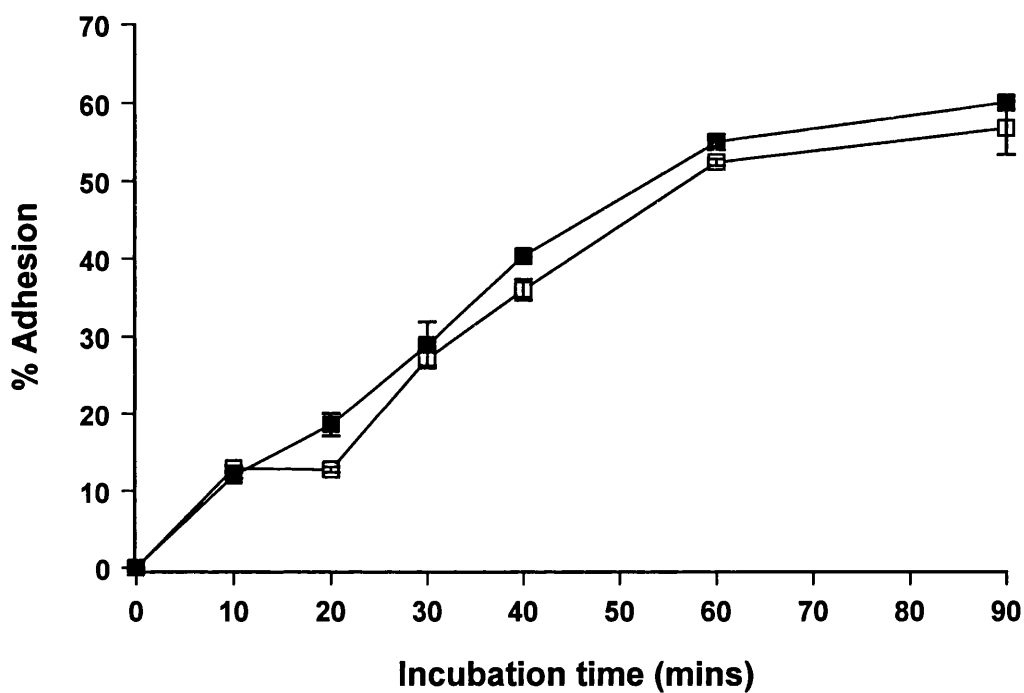
Adhesion of the C8161 cells to matrigel was not altered on treatment of the tumour cells with  $10^{-6}$ M RA (Figure 7E). 12% of untreated C8161 cells adhered to the matrigel by 10min. Adherence was increased to 27% by 30min, and to 50% by 90min.



**Figure 7E.** Adhesion of untreated C8161 cells (□) and  $10^{-6}$ M RA-pretreated C8161 cells (■) to matrigel. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

*Time point adhesion assays analysing adherence of A375 cells with and without  $10^{-6}$ M RA pretreatment to basement membrane (Matrigel).*

Adherence of the A375 cells to matrigel was unaltered with 4 days pretreatment of  $10^{-6}$ M RA (Figure 8). 13% of untreated tumour cells adhered by 10min, increasing to 27% by 30min, reaching 53% by 90min.



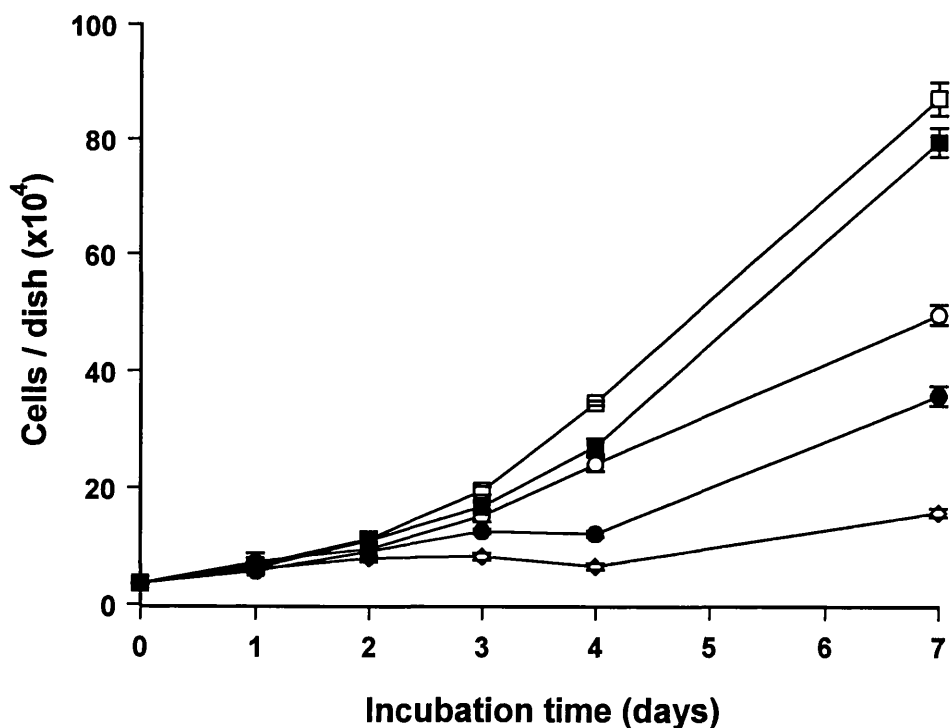
**Figure 8.** Adhesion of untreated A375 cells ( $\square$ ) and  $10^{-6}$ M RA-pretreated A375 cells ( $\blacksquare$ ) to matrigel. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

### **3.2. Pentoxifylline-induced modulation of tumour cell growth and adhesion to various ECM components**

#### *Effect of PX (10µg/ml-500µg/ml) on tumour cell growth*

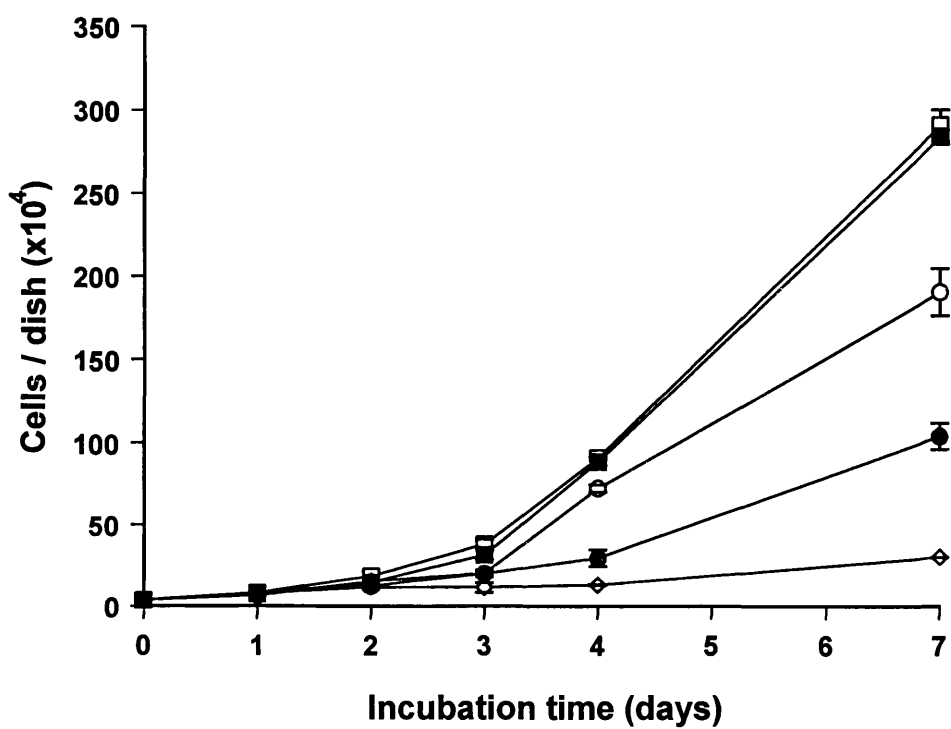
Although PX had no effect on cell morphology, it induced a dose and time-dependent decrease in growth of the Hs294T, C8161 and A375 cells (Figures 9A, 9B and 9C respectively). Viability of all the cell lines (as determined by trypan blue staining) was always greater than 90% on treatment of the tumour cells with PX, except using the highest concentration of 500µg/ml PX where viability was reduced to below 70%.

After 4 days treatment of 10µg/ml PX, a 22.0% reduction ( $p<0.05$ ) in cell numbers was observed in the Hs294T cell line (Figure 9A). Cell numbers were further reduced using 100µg/ml PX by 30.8% ( $p<0.05$ ) in the Hs294T cell line after 4 days pretreatment. Using 250µg/ml PX, cell numbers were reduced by 65.4% ( $p<0.0005$ ) (Figure 9A).



**Figure 9A.** Effect of PX (□ control; ■ 10µg/ml; ○ 100µg/ml; ● 250µg/ml; ◇ 500µg/ml) on Hs294T cell numbers. Data represents the average  $\pm$  SEM of triplicate samples.

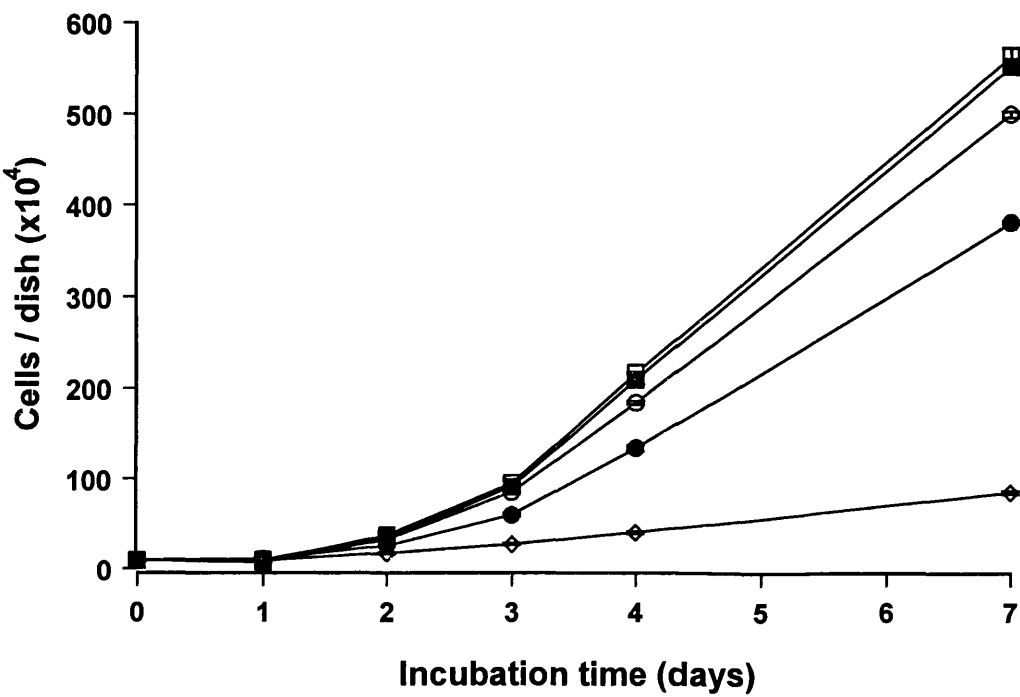
After 4 days treatment of the C8161 cell line with 10µg/ml PX, cell numbers were not significantly altered. However, using 100µg/ml PX, C8161 cell numbers were reduced by 20.3% ( $p<0.05$ ) and by 67.5% ( $p<0.01$ ) using 250µg/ml PX (Figure 9B). Pretreatment of the tumour cells with 500µg/ml PX reduced viability to below 70%.



**Figure 9B.** Effect of PX (□ control; ■ 10µg/ml; ○ 100µg/ml; ● 250µg/ml; ◇ 500µg/ml) on C8161 cell numbers. Data represents the average  $\pm$  SEM of triplicate samples.



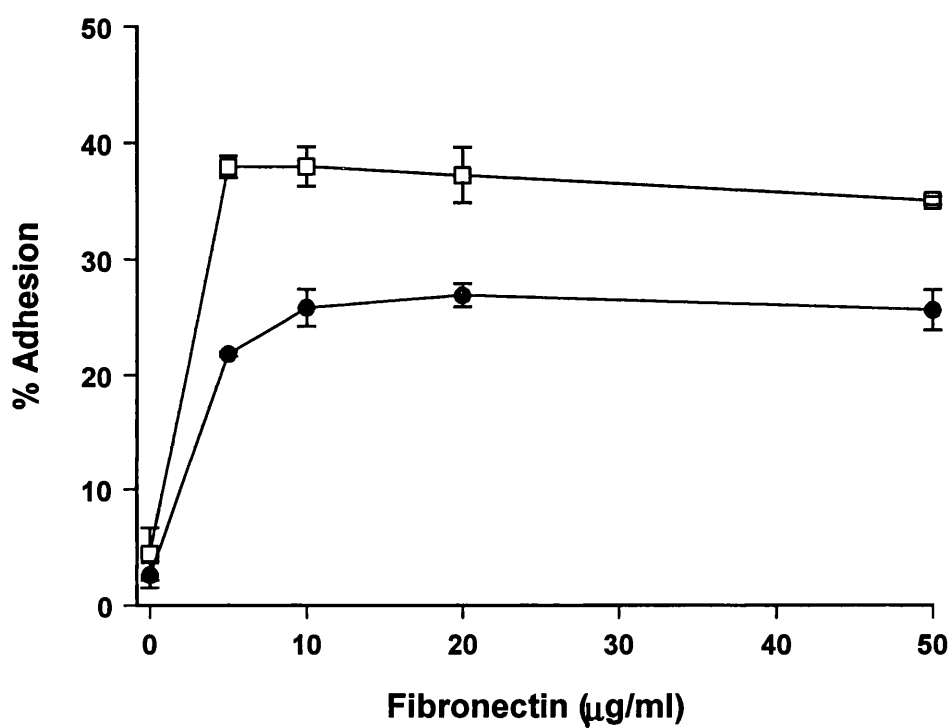
After 4 days treatment of the A375 cell line using 100µg/ml PX, cell numbers were reduced by 10.4% ( $p<0.001$ ) and by 37.2% ( $p<0.001$ ) using 250µg/ml PX (Figure 9C). Pretreatment of the tumour cells with 500µg/ml PX reduced viability to below 70%.



**Figure 9C.** Effect of PX (□ control; ■ 10µg/ml; ○ 100µg/ml; ● 250µg/ml; ◇ 500µg/ml) on A375 cell numbers. Data represents the average  $\pm$  SEM of triplicate samples.

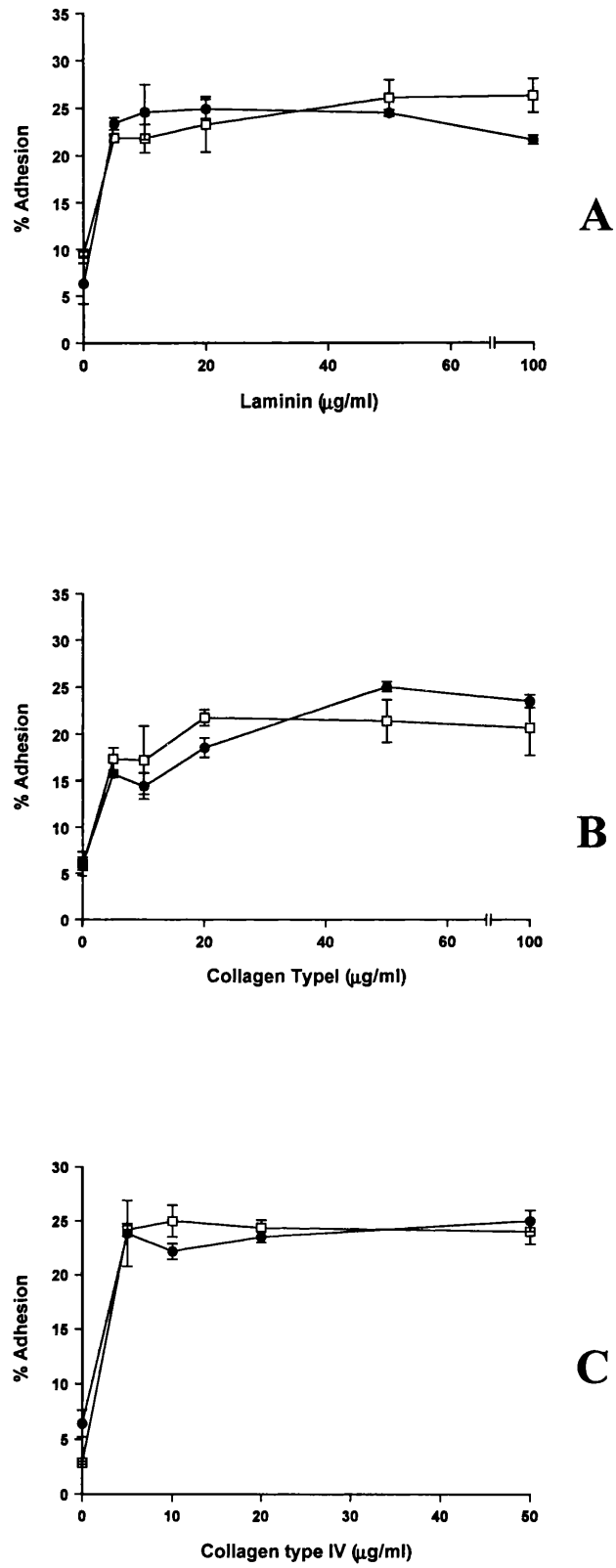
*Determining optimum concentrations of ECM components for the time-point adhesion assays of C8161 cells pretreated with 250µg/ml PX.*

Adhesion of the PX-treated C8161 cells to fibronectin was reduced at all concentrations of fibronectin tested, with a reduction of 32% and 28% using 10µg/ml and 20µg/ml fibronectin respectively (Figure 10). 10µg/ml fibronectin was selected as the optimum concentration used in the time-point adhesion assay.



**Figure 10.** Adhesion of C8161 cells without (□) and with (●) 4 days pretreatment of 250µg/ml PX to a range of concentrations of fibronectin. Values are represented as the average ± SEM of triplicate experimental dishes.

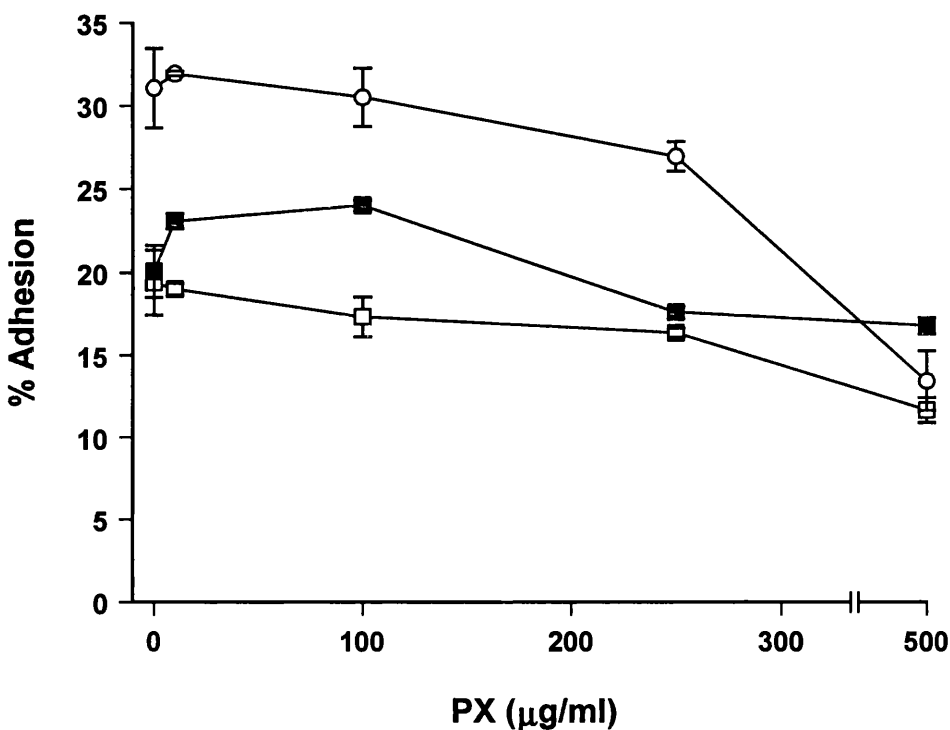
Adhesion of C8161 cells pretreated with 250µg/ml PX to various concentrations of laminin, collagen type 1 and type IV was not altered (Figures 11A, 11B and 11C respectively). Therefore, the optimum concentrations of each substrate used in the time point adhesion assays was kept the same as those used in the time-point adhesion assays of RA-treated cells (20µg/ml laminin; 10µg/ml collagen type 1; 5µg/ml collagen type IV).



**Figure 11.** Adhesion of C8161 cells without ( $\square$ ) and with ( $\bullet$ ) 4 days pretreatment of 250 $\mu\text{g/ml}$  PX to a range of concentrations A) laminin; B) collagen type I; C) collagen type IV. Values are represented as the average  $\pm$  SEM of triplicate experimental dishes.

*Effect of PX (10µg/ml - 500µg/ml) on adherence of the C8161 cells to ECM components*

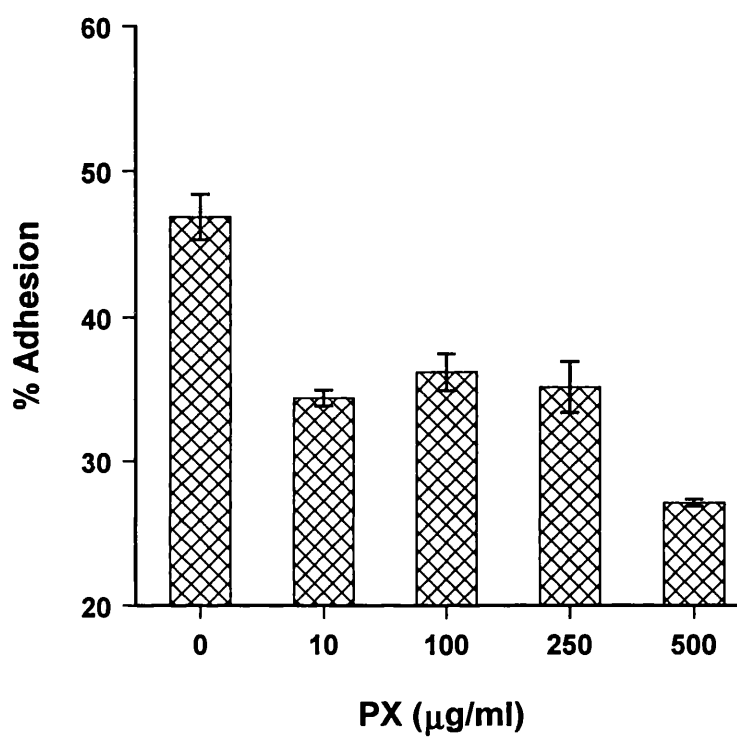
Adherence of the C8161 cells pretreated with 10µg/ml - 250µg/ml did not alter adherence to laminin, collagen type 1 and type IV (Figure 12). A significant reduction in adherence to collagen type 1 and type IV was observed pretreating cells with 500µg/ml PX, but at this concentration, viability of the cells was greatly reduced to below 70%.



**Figure 12.** Adherence of C8161 cells pretreated with PX (10µg/ml - 500µg/ml) to collagen type IV (□); laminin (■) and collagen type I (○). Values represent the average ± SEM of triplicate experimental dishes where the value at zero concentration was determined by adhesion of untreated cells to substrate.

*Effect of PX (10µg/ml - 500µg/ml) on adherence of the C8161 cells to fibronectin*

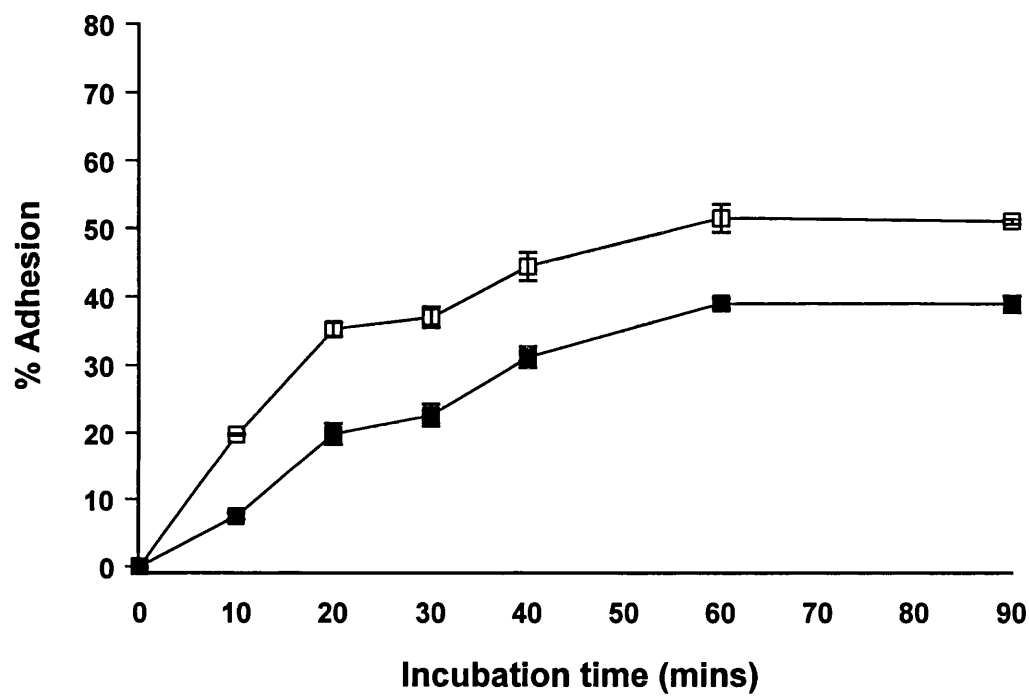
PX reduced adherence of the C8161 cells by 26.5% ( $p<0.05$ ) using 10µg/ml PX which was similar at the higher concentration of 250µg/ml where adhesion was reduced by 24.9% ( $p<0.05$ ) (Figure 13).



**Figure 13.** Effect of PX (10µg/ml- 500µg/ml) on adherence of C8161 cells to fibronectin. Values represent the average  $\pm$  SEM of triplicate experimental dishes where the value at zero concentration was determined by adhesion of untreated cells to fibronectin.

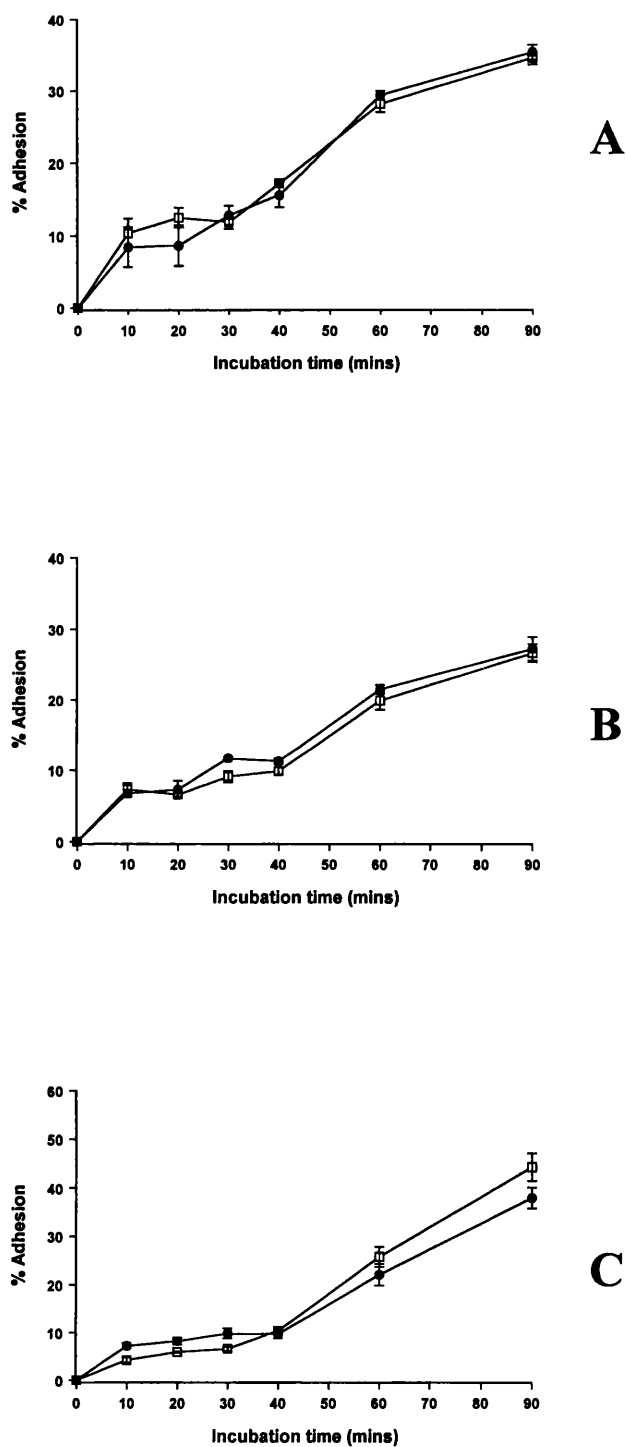
*Time point adhesion assays analysing adherence of C8161 cells with and without 250µg/ml PX pretreatment to various ECM components.*

PX pretreatment (250µg/ml) of the C8161 cell line for a 4 day period caused a significant (24.2%) reduction in adherence of the tumour cells to fibronectin by 30 min ( $p<0.05$ ) with a similar reduction of 29.8% ( $p<0.05$ ) maintained at the 40min time period (Figure 14).



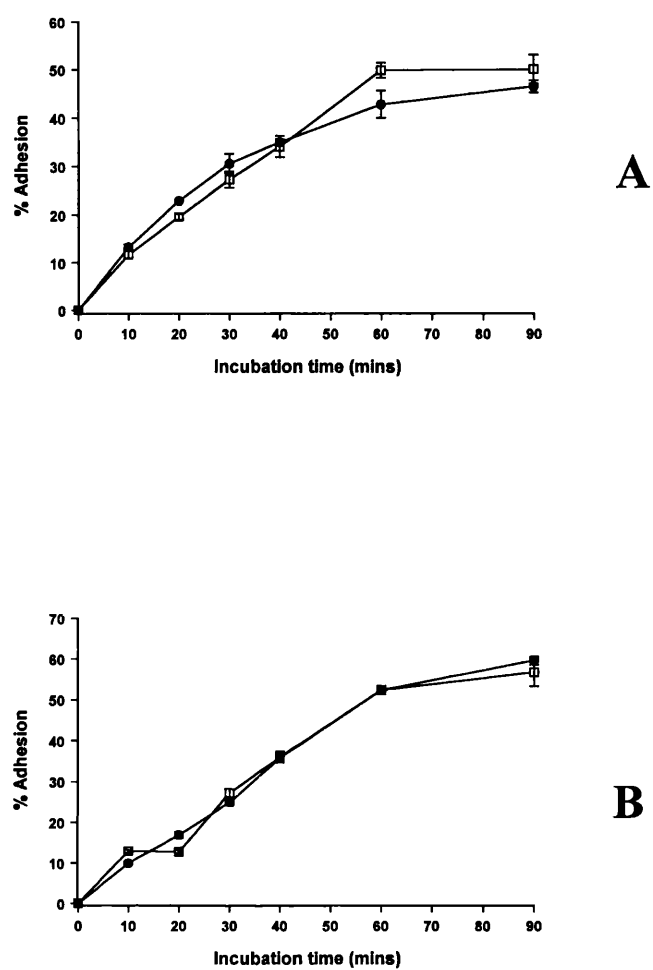
**Figure 14.** Adherence of C8161 cells without (□) and with (●) 4 days pretreatment of 250µg/ml PX to fibronectin. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

Adhesion of C8161 cells pretreated for 4 days with PX (250µg/ml) to laminin, collagen type I and IV was not altered in comparison to untreated tumour cells (Figures 15A, B and C respectively).



**Figure 15.** Adherence of C8161 cells to A) laminin; B) collagen type I; C) collagen type IV without (□) and with (●) 4 days pretreatment of 250µg/ml PX. Values represent the average  $\pm$  SEM of triplicate experimental dishes.

PX (250µg/ml) failed to alter adhesion of the C8161 cells and the A375 cells to basement membrane matrigel (Figures 16A and 16B respectively).



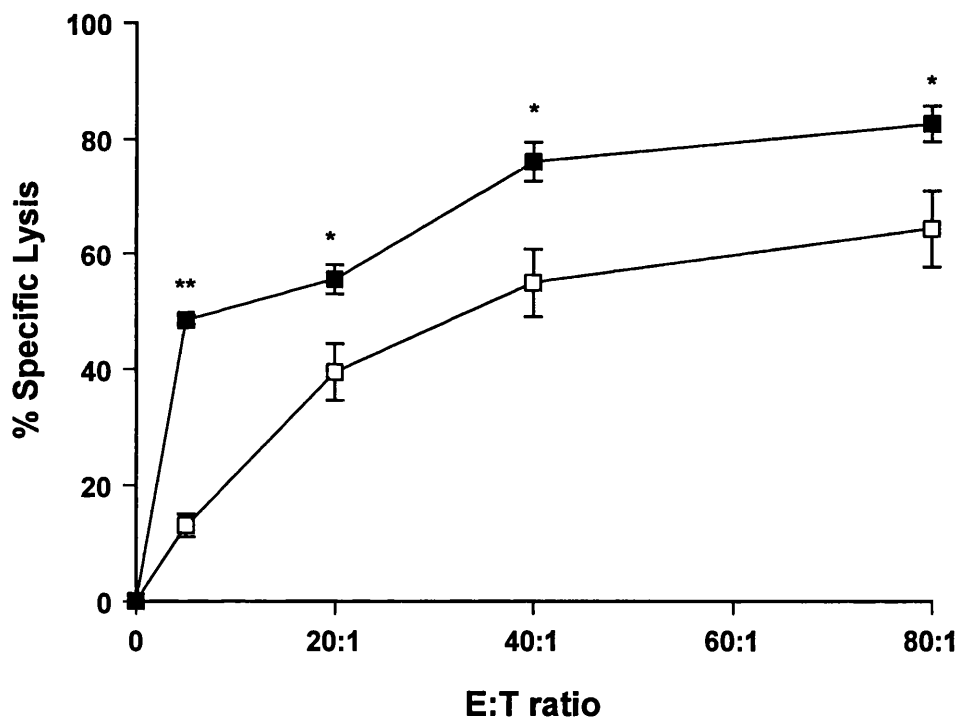
**Figure 16.** Adherence of A) C8161 cells and B) A375 cells without (□) and with (●) 4 days pretreatment of 250µg/ml PX to basement membrane matrigel. Values represent the average ± SEM of triplicate experimental dishes.



**3.3. Retinoid-modulation of tumour LAK cell-mediated lysis**

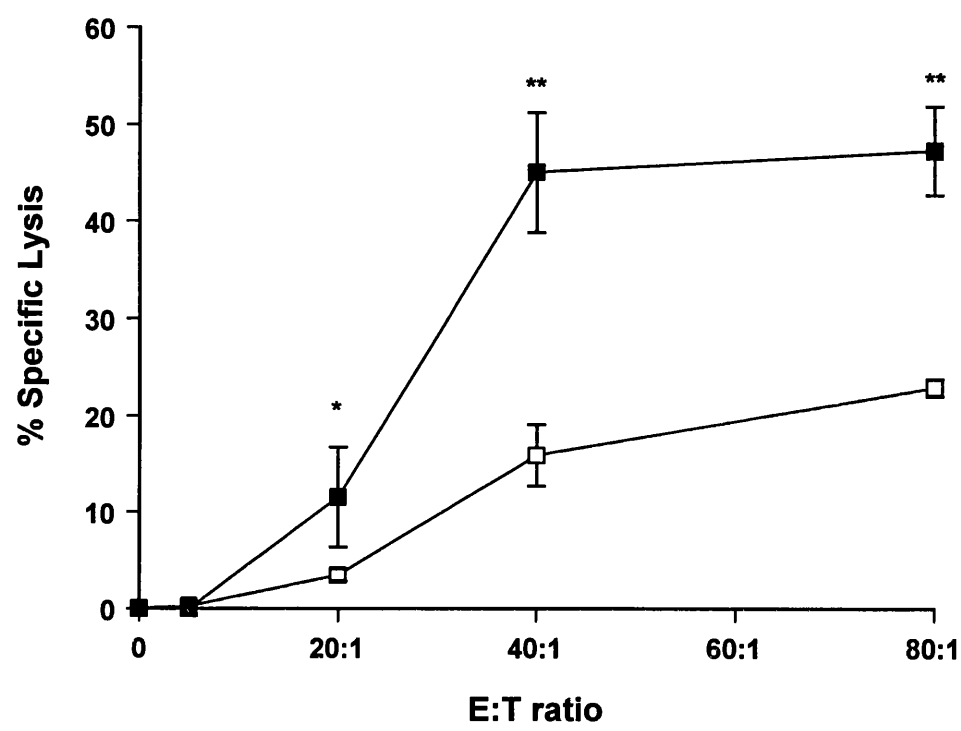
*Effect of RA on lysis of the human melanoma cell lines.*

Unactivated peripheral blood lymphocytes were unable to lyse any of the three cell lines tested (data not shown).  $10^{-6}$ M RA-treated Hs294T and C8161 cells incubated with LAK cells for a period of 4hr were found to become more susceptible to lysis than untreated control cells (Figures 17A and 17B respectively), whereas RA was unable to alter lysis of the A375 cells (Figure 17C). In the Hs294T cell line (Figure 17A), lysis was increased 3.7-fold at the 5:1 ratio from 13.1% to 48.4% ( $p<0.005$ ), from 39.5% to 55.5% at the 20:1 ratio (1.4-fold), and from 54.9% to 76.1% at the 40:1 ratio (1.4-fold) with  $p$  values $<0.05$ .



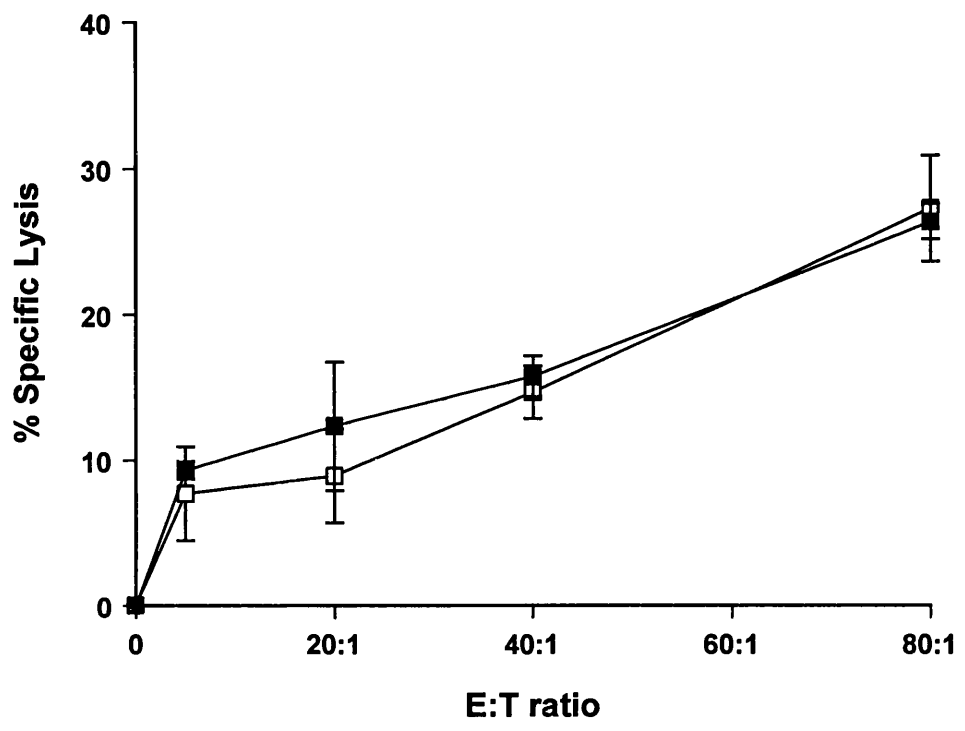
**Figure 17A.** IL2-activated PBL lysis of Hs294T cells pretreated with (■) and without (□)  $10^{-6}$ M RA. Values are respresented as the average  $\pm$  SEM of five samples where \* $P<0.05$  and \*\* $P<0.005$ .

A similar pattern was observed using the C8161 cell line (Figure 17B), where lysis was increased 3.3-fold from 3.5% to 11.5% at the 20:1 ratio ( $p<0.05$ ) with similar increases of 2.8-fold ( $p<0.005$ ) and 2.1-fold ( $p<0.005$ ) at the 40:1 and 80:1 ratios respectively.



**Figure 17B.** IL2-activated PBL lysis of C8161 cells pretreated with (■) and without (□)  $10^{-6}$ M RA. Values are represented as the average  $\pm$  SEM of five samples where \* $P<0.05$  and \*\* $P<0.005$ .

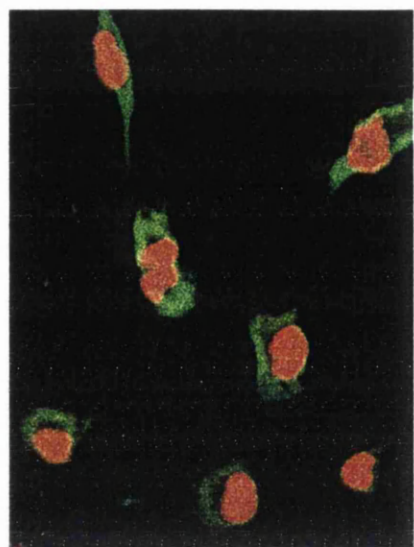
Pretreatment of the A375 cells with  $10^{-6}$  M RA failed to alter tumour cell lysis (Figure 17C).



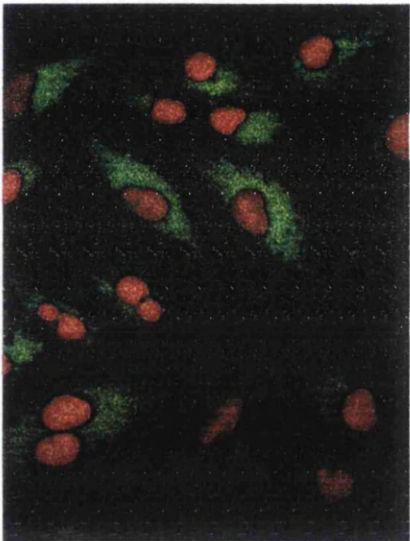
**Figure 17C.** IL2-activated PBL lysis of A375 cells pretreated with (■) and without (□)  $10^{-6}$  M RA. Values are represented as the average  $\pm$  SEM of five samples.

### *Confocal Analysis and Flow Cytometric Analysis of mICAM-1 Expression*

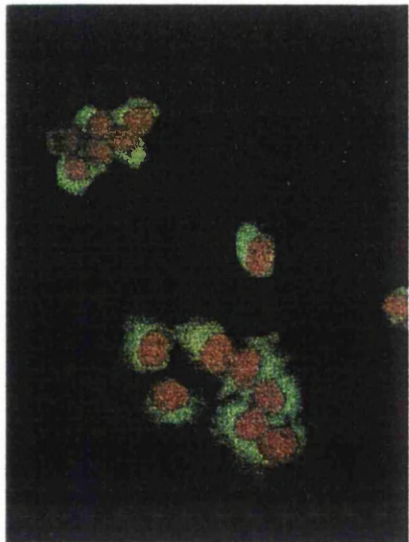
All three cell lines were found to express mICAM-1 (Figures 18A, B and C for C8161, Hs294T and A375 cells respectively) and MHC class 1 (Figures 19A, B and C for C8161, Hs294T and A375 cells respectively). Negative controls for MHC class 1 staining are shown in Figure 20. Flow cytometric analysis of membrane ICAM-1 expression of C8161 cells and Hs294T cells (Figures 21A and 21B respectively) exposed for 4 days to RA at concentrations of  $10^{-10}$  M -  $10^{-7}$  M did not significantly alter membrane ICAM-1 expression but a positive shift in intensity became apparent at concentrations of  $10^{-6}$  M -  $10^{-5}$  M RA. This positive shift in fluorescent intensity corresponds to an increase in mICAM-1 levels. Using the A375 cells, pretreatment with  $10^{-10}$  M -  $10^{-7}$  M RA marginally induced mICAM-1 expression (Figure 21C) which further increased on treatment with  $10^{-6}$  M -  $10^{-5}$  M RA.



A

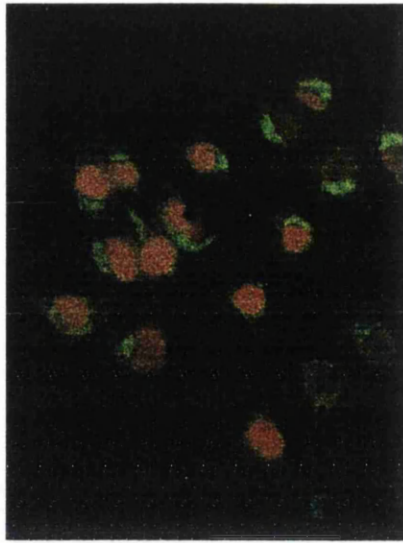


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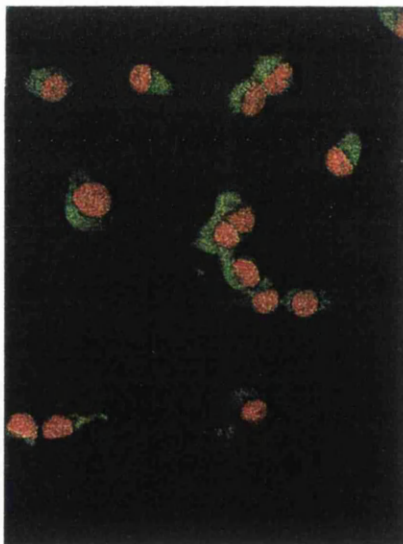
**Figure 19.** Expression of ICAM-1 in A) C8161; B) Hs294T; C) A375 cells.



A

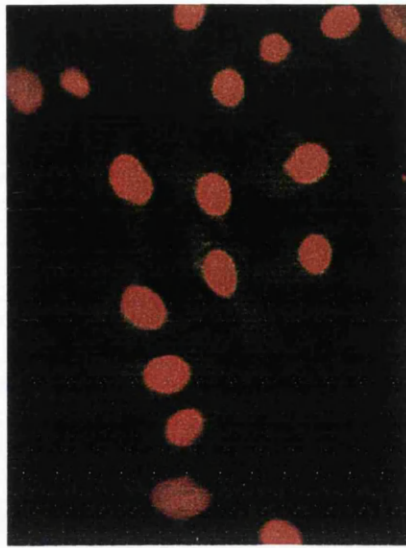


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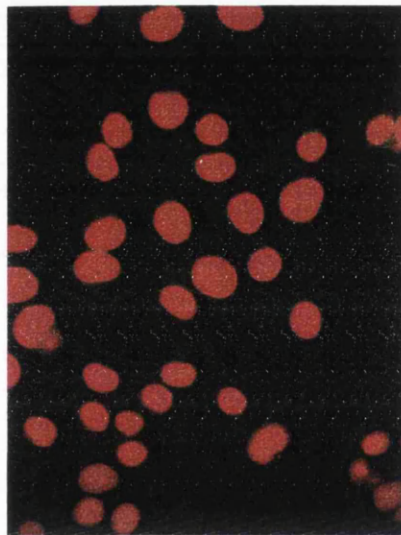


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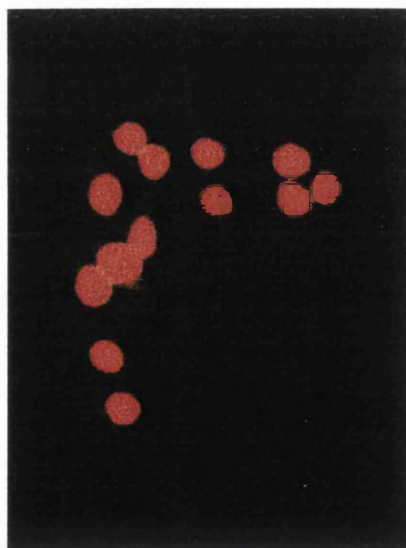
**Figure 20.** Expression of MHC class 1 in A) C8161; B) Hs294T ; C) A375 cells.



A



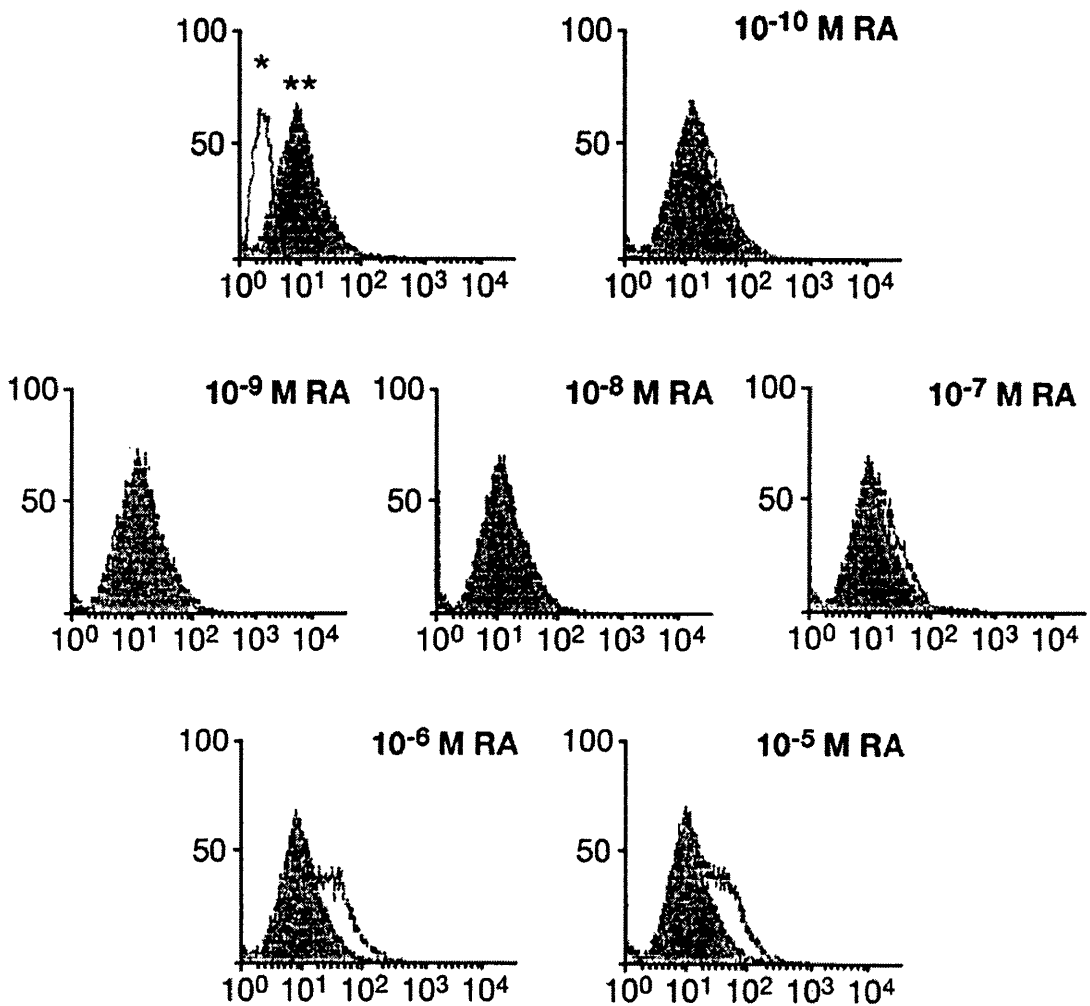
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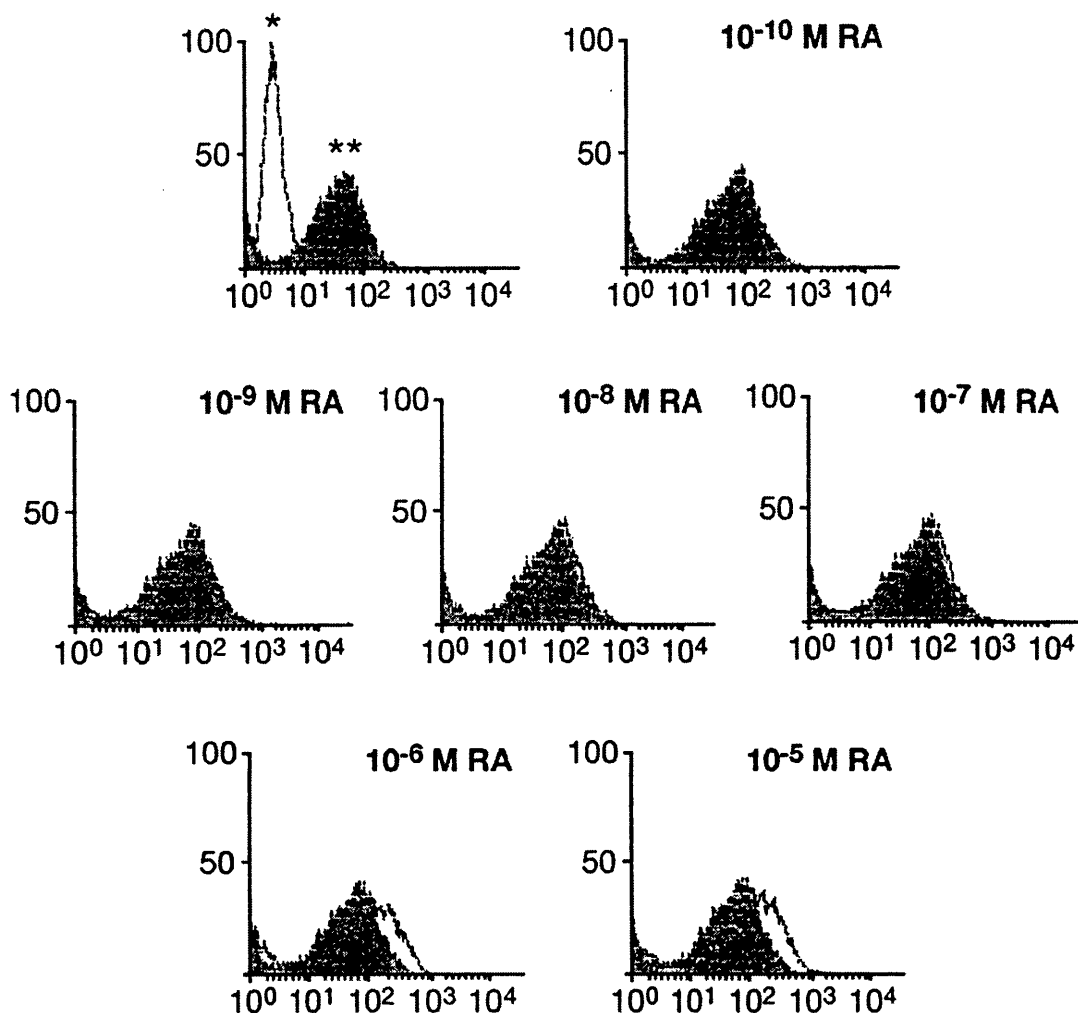
**Figure 21.** Staining of A) C8161; B) Hs294T; C) A375 cells with secondary FITC-conjugated antibody used in MHC Class 1 staining.

*Flow Cytometric analysis of mICAM-1 Expression*

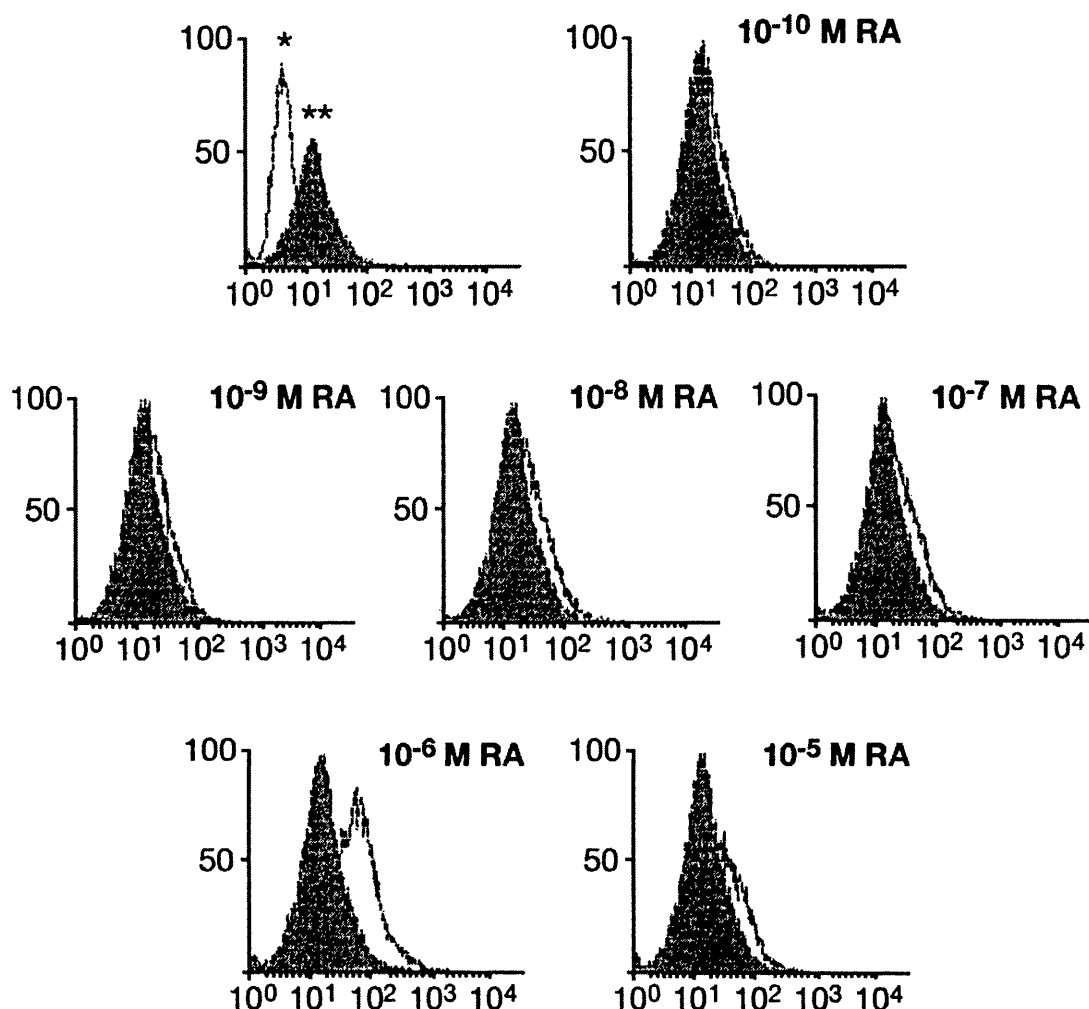


**Figure 21A.** Flow cytometric analysis of membrane ICAM-1 expressed on C8161 cells. Tumour cells were incubated for 4 days in the presence (unshaded area) and absence (shaded area) of RA. mICAM-1 was detected using FITC-labelled anti-human ICAM-1 antibody (\*\*). Sample minus antibody was used to determine background fluorescent intensity denoted by \*. The x-axis represents log fluorescent intensity and the y-axis, cell numbers.



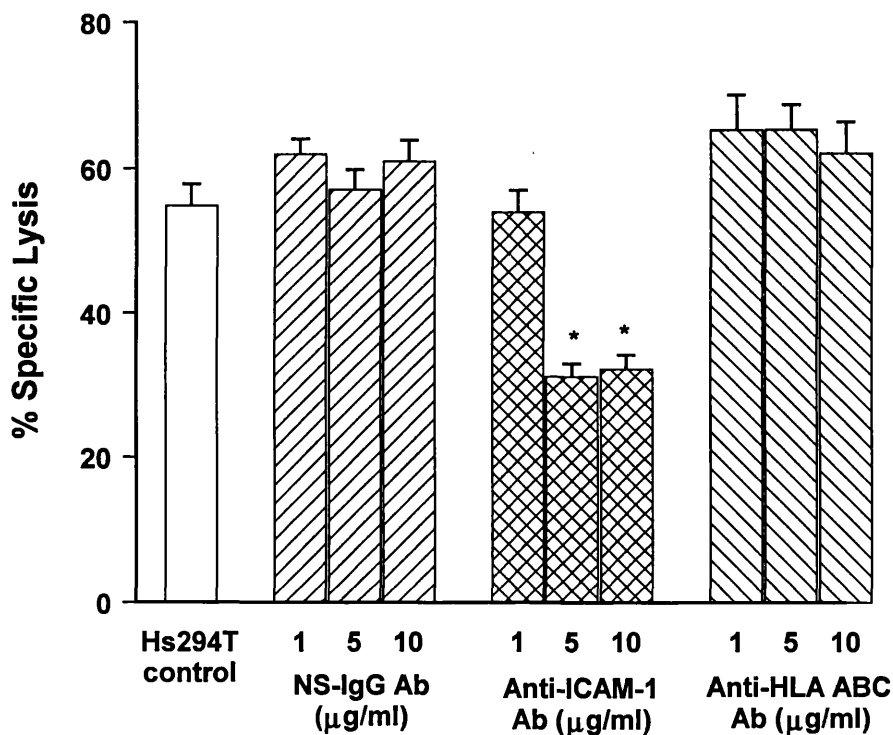


**Figure 21B.** Flow cytometric analysis of membrane ICAM-1 expressed on Hs294T cells. Tumour cells were incubated for 4 days in the presence (unshaded area) and absence (shaded area) of RA. mICAM-1 was detected using FITC-labelled anti-human ICAM-1 antibody (\*\*). Sample minus antibody was used to determine background fluorescent intensity denoted by \*. The x-axis represents log fluorescent intensity and the y-axis, cell numbers.



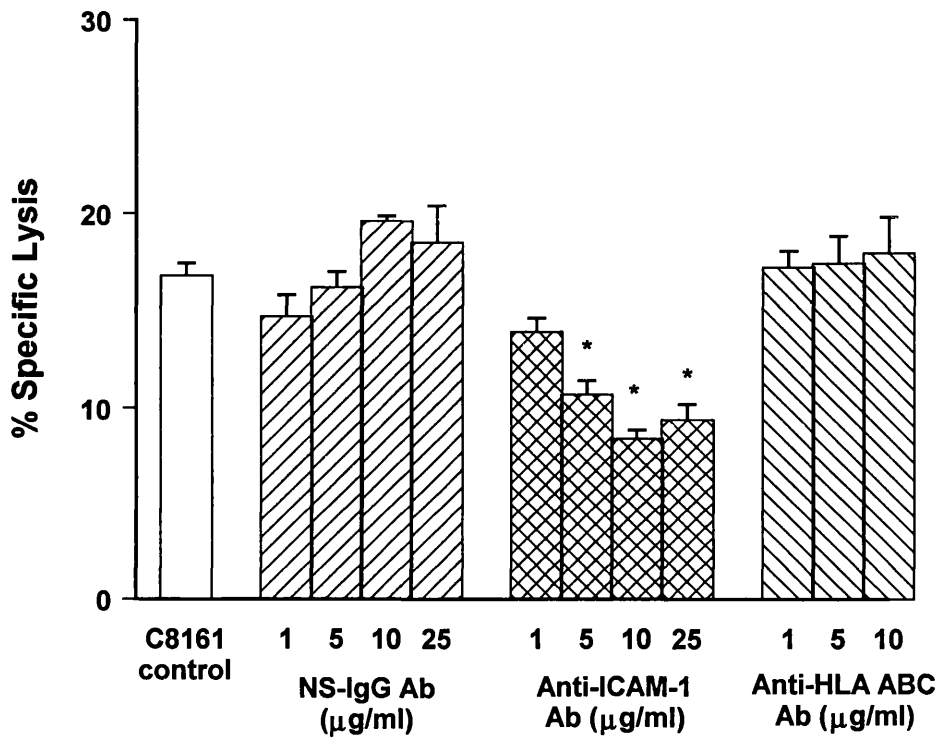
**Figure 21C.** Flow cytometric analysis of membrane ICAM-1 expressed on A375 cells. Tumour cells were incubated for 4 days in the presence (unshaded area) and absence (shaded area) of RA. mICAM-1 was detected using FITC-labelled anti-human ICAM-1 antibody (\*\*). Sample minus antibody was used to determine background fluorescent intensity denoted by \*. The x-axis represents log fluorescent intensity and the y-axis, cell numbers.

For all comparisons, lysis on addition of blocking ICAM-1 antibody was compared to a non-specific IgG control antibody. Using the Hs294T cell line (Figure 22A), blocking ICAM-1 at the optimal concentration of 5µg/ml significantly reduced lysis of control cells 1.8-fold from 57.1% to 31.1% ( $p<0.001$ ) at the 40:1 ratio. This reduction was also observed using 10µg/ml ICAM-1 antibody which reduced lysis 1.9-fold ( $p<0.001$ ). Anti-HLA-ABC antibody over a range of concentrations had no effect on Hs294T cell lysis (Figure 22A).



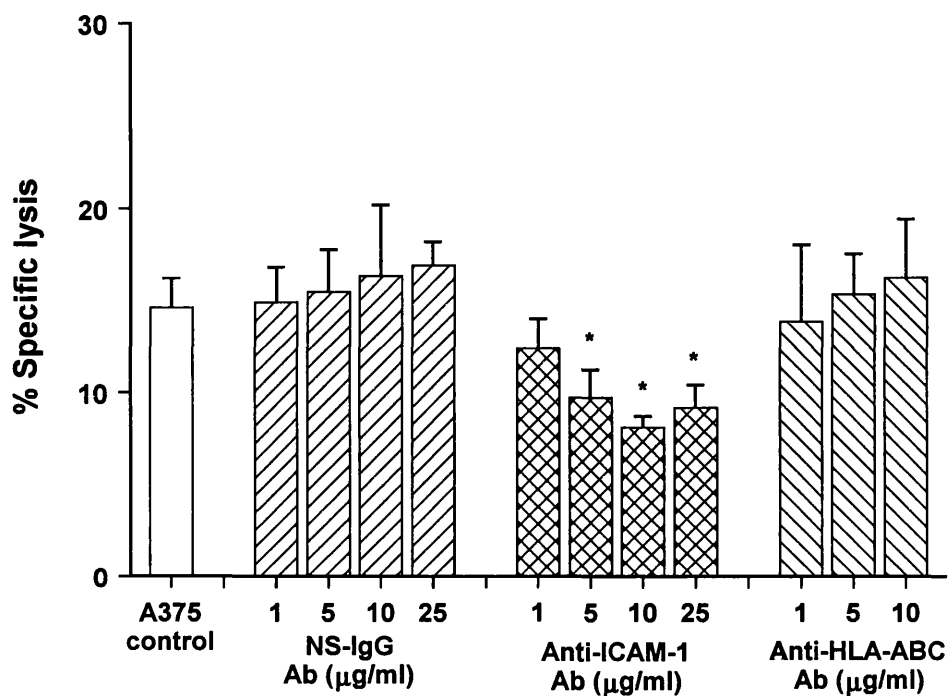
**Figure 22A.** Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back-hatched bar) on the lysis of Hs294T cells (open bar) at an E:T ratio of 40:1. Non specific IgG antibody was also included as a control (forward-hatched bar). Values are represented as the average  $\pm$  SEM of five samples where \* $P<0.0005$  and represents comparison of control IgG with specific antibody.

In the C8161 cells, addition of 5µg/ml of ICAM-1 antibody at the 40:1 ratio (Figure 22B) caused a highly significant 1.5-fold decrease in lysis of control C8161 cells (from 16.2% to 10.7%,  $p<0.0005$ ). Optimum inhibition was achieved using 10µg/ml of ICAM-1 antibody which reduced lysis from 19.6% to 8.4% (a 2.3-fold difference,  $p<0.0005$ ). This inhibition was maintained at the higher concentration of antibody. As with the Hs294T cells, addition of anti-HLA ABC antibody had no effect on lysis of the C8161 cells (Figure 22B).



**Figure 22B.** Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back-hatched bar) on the lysis of C8161 cells (open bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward-hatched bar). Values are represented as the average  $\pm$  SEM of five samples where \* $P<0.0005$  and represents comparison of control IgG with specific antibody.

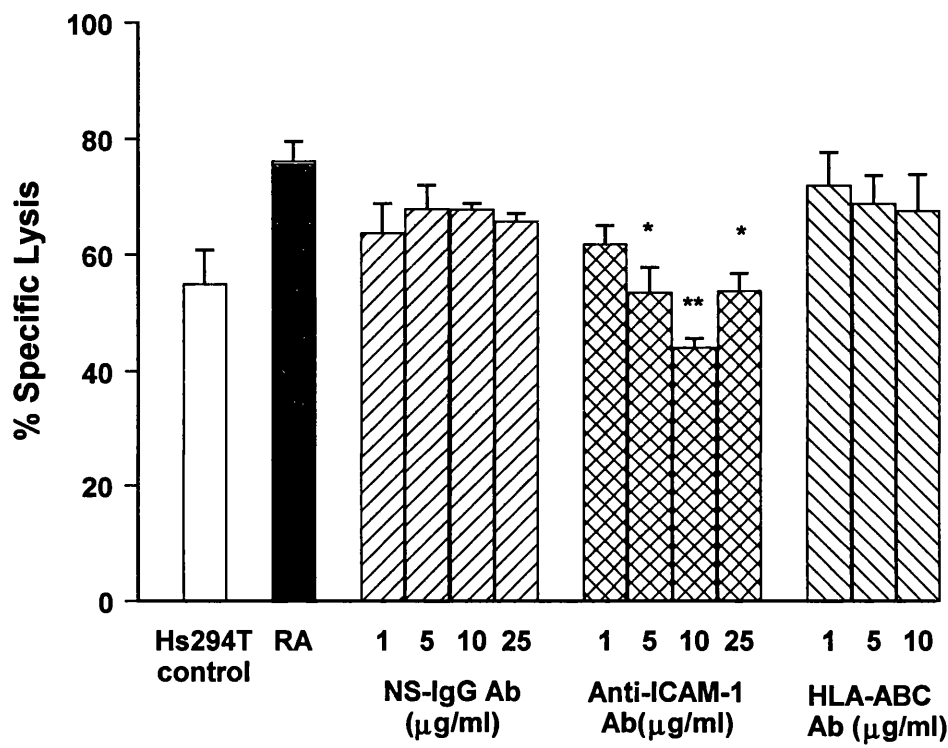
Addition of blocking ICAM-1 antibody (5 $\mu$ g/ml) significantly reduced lysis of the A375 cells 1.6-fold ( $p<0.001$ ), and by 2.0-fold using 10 $\mu$ g/ml antibody. Blocking HLA-ABC antibody had no effect on cell lysis.



**Figure 22C.** Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back-hatched bar) on the lysis of A375 cells (open bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward-hatched bar). Values are represented as the average  $\pm$  SEM of five samples where \* $P<0.0005$  and represents comparison of control IgG with specific antibody.

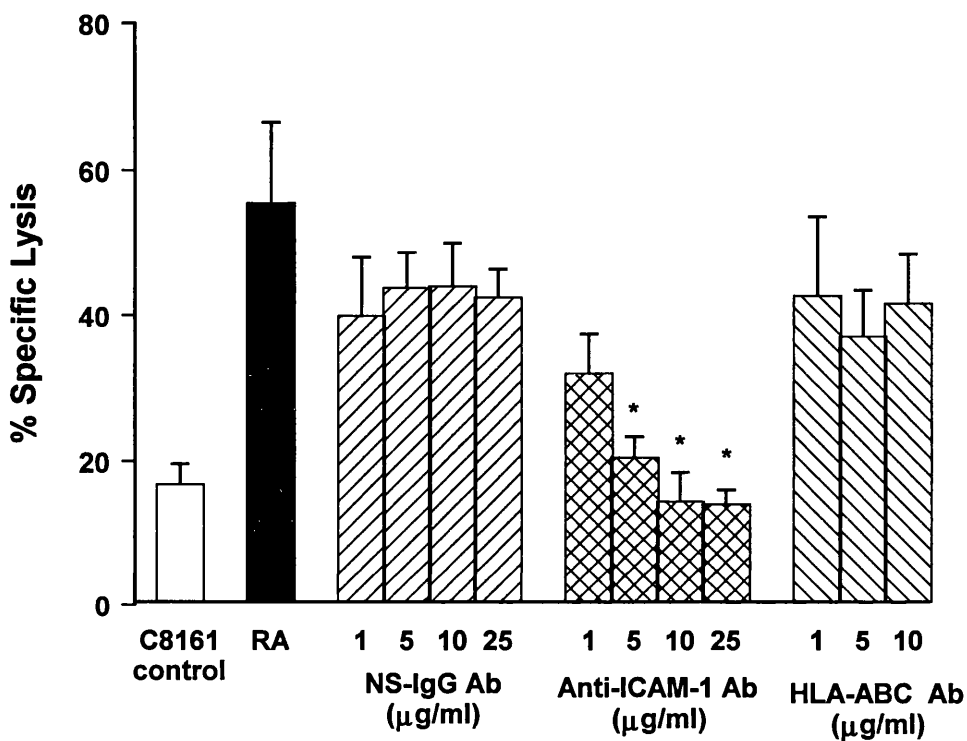
*Effect of anti-ICAM-1 and HLA-ABC antibody on LAK-cell mediated lysis of RA pretreated tumour cells.*

On treatment of Hs294T cells with  $10^{-6}$  M RA (Figure 23A) lysis was significantly reduced using ICAM-1 antibody at 5 $\mu$ g/ml which caused a 1.3-fold reduction in lysis from 67.8% to 53.4% ( $p<0.05$ ). Similar reductions were observed at all other antibody concentrations tested. Anti-HLA ABC antibody had no effect on LAK cell mediated lysis (Figure 23A).



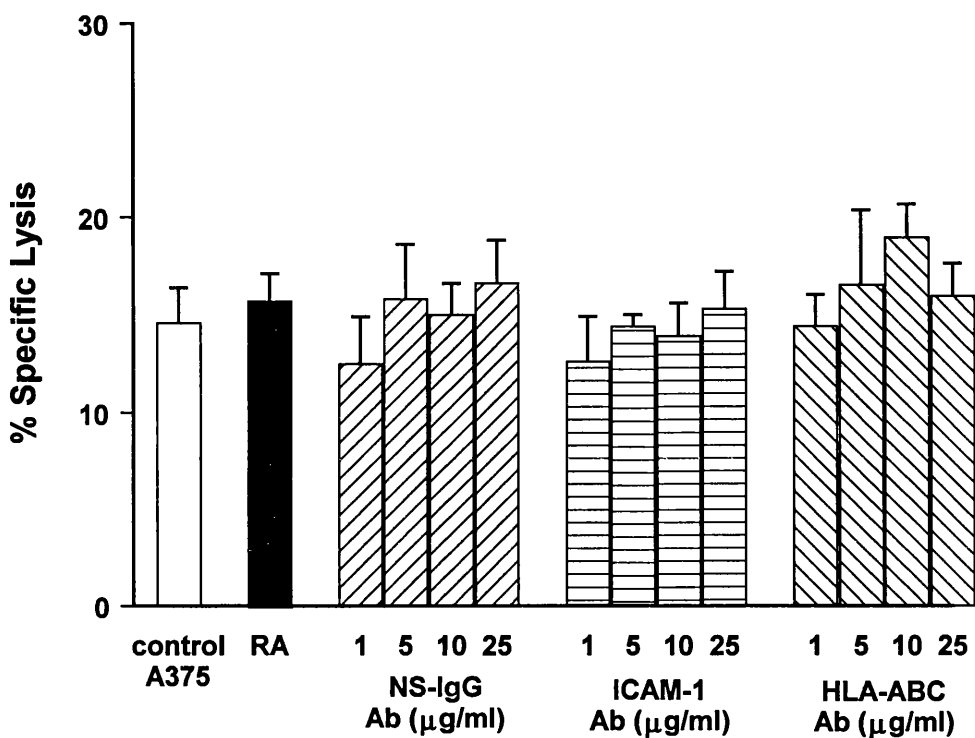
**Figure 23A.** Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back-hatched bar) on the lysis of Hs294T cells pretreated for 4 days with  $10^{-6}$  M RA (solid bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward-hatched bar). Values are represented as the average  $\pm$  SEM of five samples where \* $P<0.05$  and \*\* $P<0.005$ , and represents comparison of control IgG with anti-ICAM-1 antibody.

A similar pattern was also observed in RA-treated C8161 cells (Figure 23B) where lysis was reduced at 5µg/ml ICAM-1 antibody from 43.7% to 20.5% (p<0.005). Optimum inhibition was also achieved using 10µg/ml ICAM antibody which induced a 3-fold reduction in lysis of the cells from 44.0% to 14.4% (p<0.005) which was similar at the higher concentration of antibody. Lysis was not altered on addition of HLA-ABC antibody.



**Figure 23B.** Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back-hatched bar) on the lysis of C8161 cells pretreated for 4 days with  $10^{-6}$ M RA (solid bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward-hatched bar). Values are represented as the average  $\pm$  SEM of five samples where \*P<0.005 and represents comparison of control IgG with anti-ICAM-1 antibody.

Blocking ICAM-1 and HLA-ABC antibodies were unable to alter lysis of RA-treated A375 cells (Figure 23C).

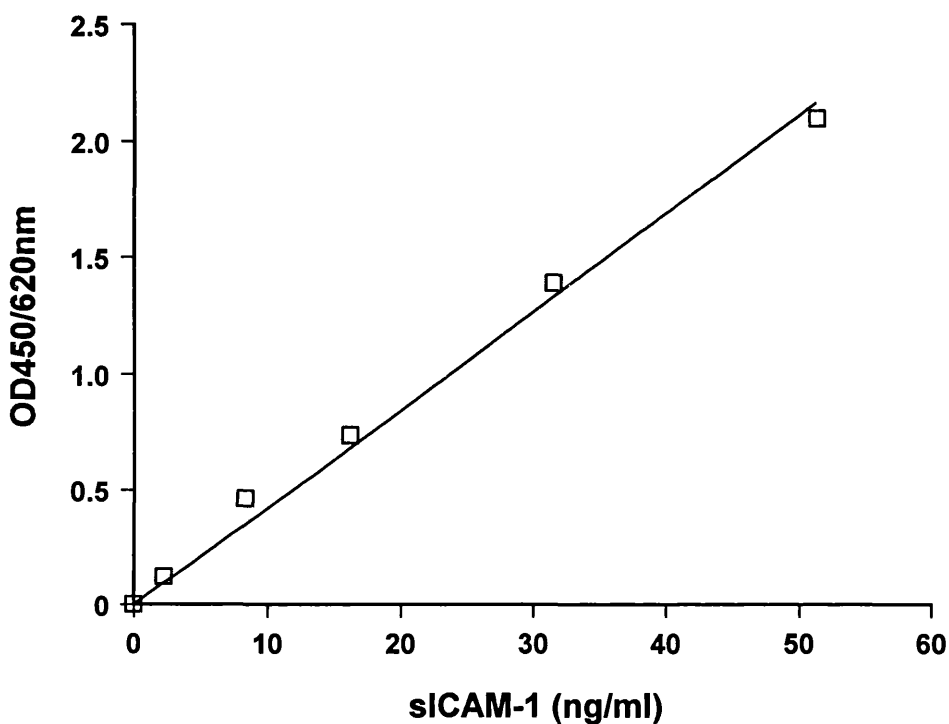


**Figure 23C.** Effect of blocking ICAM-1 antibody (horizontal hatched bar) and blocking HLA-ABC antibody (back-hatched bar) on the lysis of A375 cells pretreated for 4 days with  $10^{-6}$  M RA (solid bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward-hatched bar). Values are represented as the average  $\pm$  SEM of five samples and represents comparison of control IgG with specific antibody.



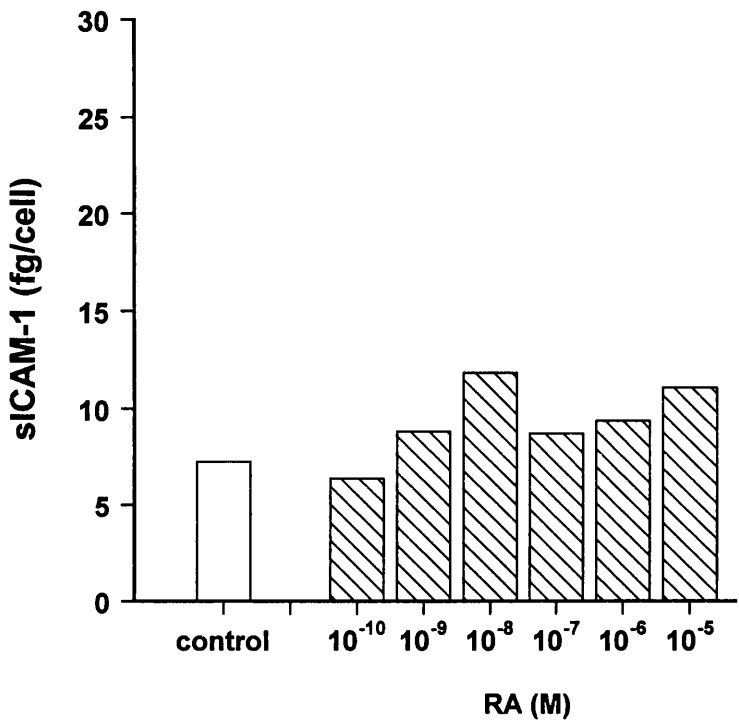
**3.4. Retinoid modulation of sICAM-1 secretion**

A standard curve was constructed from samples of known concentrations of sICAM-1 (Figure 24) to allow the unknown levels of sICAM-1 to be determined from the tumour cell supernatants.



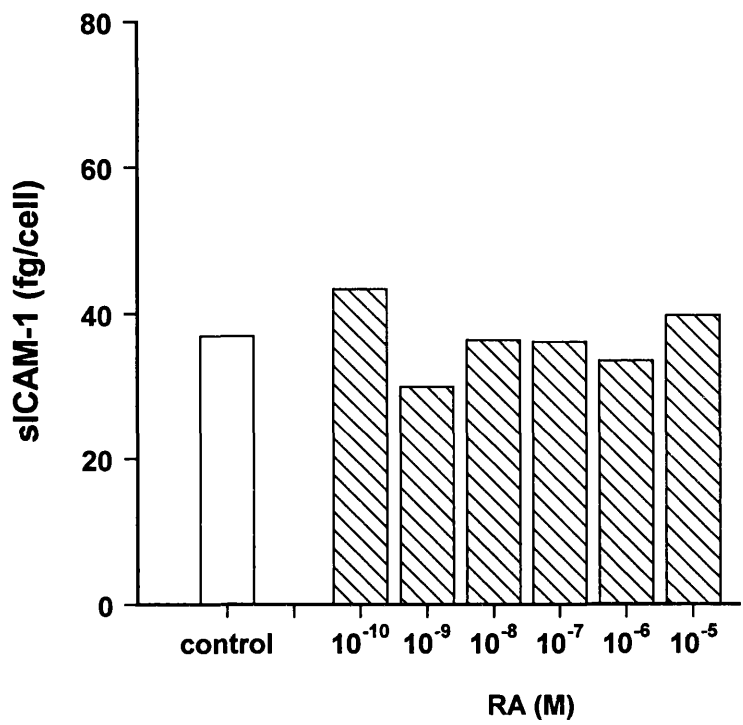
**Figure 24.** Standard curve of sICAM-1 where  $y=0.042x$ . The data represents the average of duplicate samples.

The Hs294T cell line secreted 7.2fg/cell of soluble ICAM-1 which did not change significantly following treatment with  $10^{-10}\text{M}$  -  $10^{-5}\text{M}$  RA (range 7.2fg/cell - 11.8fg/cell) (Figure 25A).



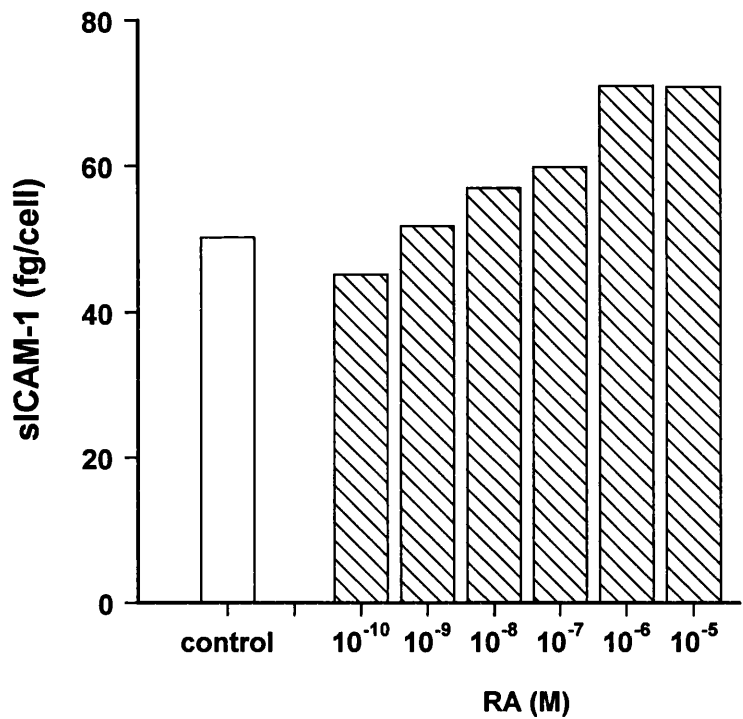
**Figure 25A.** Effect of RA on sICAM-1 secretion from the Hs294T cells. Values represent the average of duplicate samples.

C8161 cells secreted basal levels of 36.9fg ICAM-1 per cell (Figure 25B). Levels were not altered on treatment of tumour cells with  $10^{-10}\text{M}$  -  $10^{-5}\text{M}$  RA (range 29.9fg/cell - 43.4fg/cell).



**Figure 25B.** Effect of RA on sICAM-1 secretion from the C8161 cells. Values represent the average of duplicate samples.

The A375 cells secreted basal levels of sICAM-1 of 50.5fg/cell (Figure 25C). At the higher concentrations of  $10^{-6}$  M and  $10^{-5}$  M RA, sICAM-1 expression was increased to 70.9fg/cell and 70.7fg/cell respectively.



**Figure 25C.** Effect of RA on sICAM-1 secretion from the A375 cells. Values represent the average of duplicate samples.

*Addition of sICAM-1 to the cytotoxicity assays*

At the 40:1 ratio, addition of sICAM-1 to control C8161 cells did not alter their susceptibility but RA treatment of cells in combination with sICAM-1 addition, significantly reduced lysis from 76.0% to 24.3% (Table 1).

In contrast, addition of sICAM-1 at the 40:1 ratio, using control or RA-treated Hs294T did not alter tumour cell lysis (Table 1). Addition of sICAM-1 to A375 cells has not been investigated.

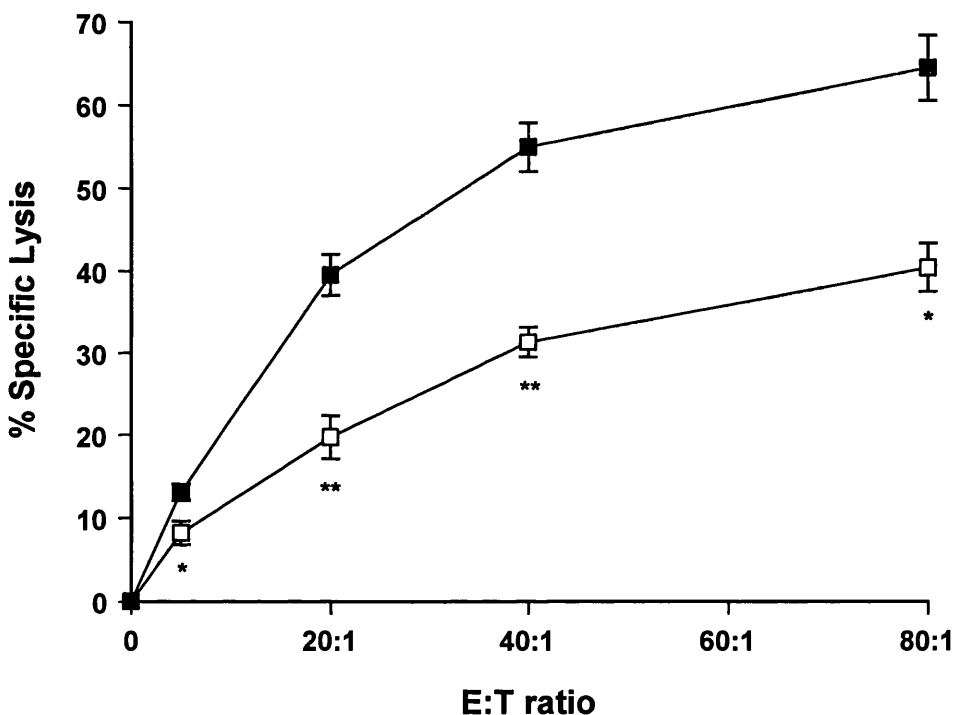
	C8161		Hs294T	
	- sICAM	+ sICAM	- sICAM	+ sICAM
control	35.6% ± 3.7	36.5% ± 2.7	46.4% ± 1.8	48.3% ± 1.1
10 <sup>-6</sup> M RA	76.0% ± 3.3	24.3% ± 3.4	80.9% ± 8.4	81.3% ± 5.9

**Table 1.** Effect of soluble ICAM-1 on the lysis of C8161 cells and Hs294T cells with and without treatment of 10<sup>-6</sup>M RA.

**3.5. Pentoxifylline modulation of tumour LAK cell-mediated lysis**

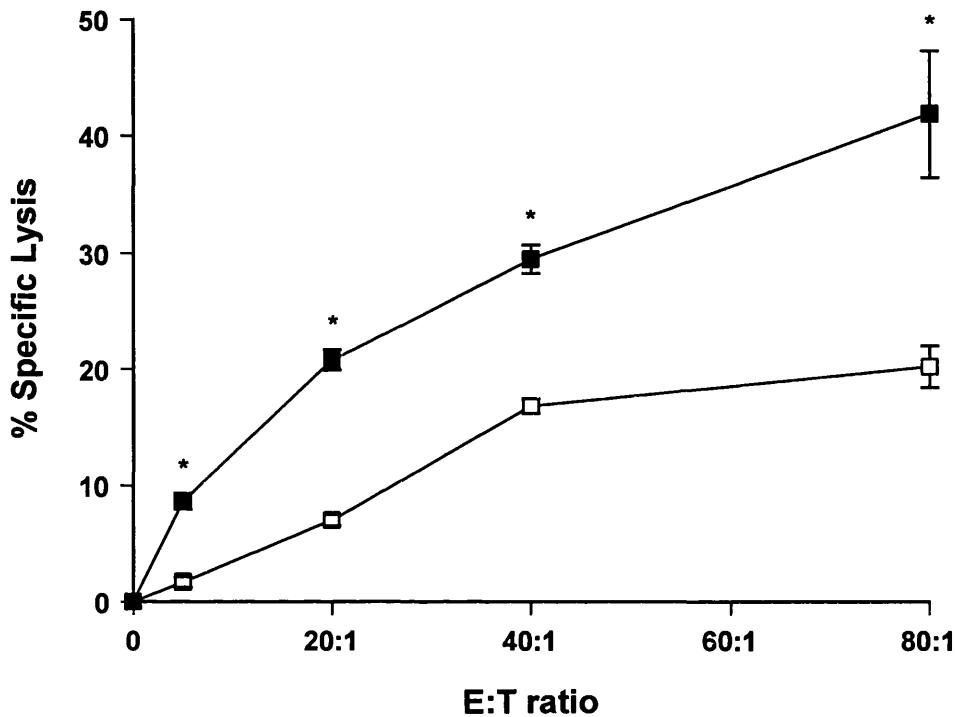
Unactivated PBL failed to induce lysis of all untreated/treated tumour cells and at all E:T ratios tested (data not shown).

Pretreatment of tumour cells for 4 days with PX (250µg/ml) induced the Hs294T cells to become less susceptible to lysis by LAK cells (Figure 26A) and had the opposite effect on the C8161 cells (Figure 26B). At the 40:1 E:T ratio, lysis of PX-treated Hs294T cells was reduced from 54.9% to 31.3% ( $p<0.005$ ) at the 40:1 ratio and from 64.4% to 40.4% ( $p<0.05$ ) at the 80:1 ratio (Figure 26A).



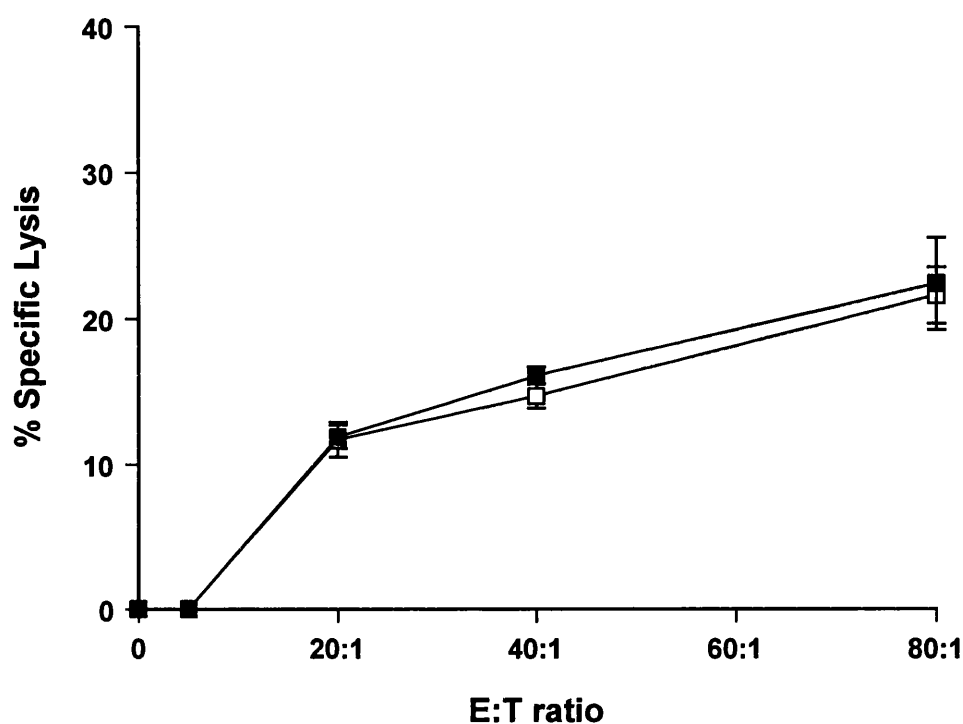
**Figure 26A.** Lysis of Hs294T cells with (□) and without (■) 4 days pretreatment of PX (250µg/ml). Data represents the average  $\pm$  SEM of five samples where \* $P<0.05$  and \*\* $P<0.005$ .

PX treatment of the C8161 cell line increased LAK cell-mediated lysis from 16.8% to 29.4% ( $p<0.0005$ ) at the 40:1 ratio and from 20.1% to 41.7% ( $p<0.0005$ ) at the 80:1 ratio (Figure 26B).



**Figure 26B.** Lysis of C8161 cells with (■) and without (□) 4 days pretreatment of PX (250µg/ml). Data represents the average  $\pm$  SEM of five samples where \* $P<0.0005$ .

4 day pretreatment of the low metastatic A375 cell line with 250µg/ml PX failed to alter LAK cell-mediated lysis of the tumour cells at all E:T ratios tested (Figure 26C).



**Figure 26C.** LAK cell lysis of A375 cells pretreated with (■) and without (□) 250µg/ml PX. Values are represented as the average ± SEM.

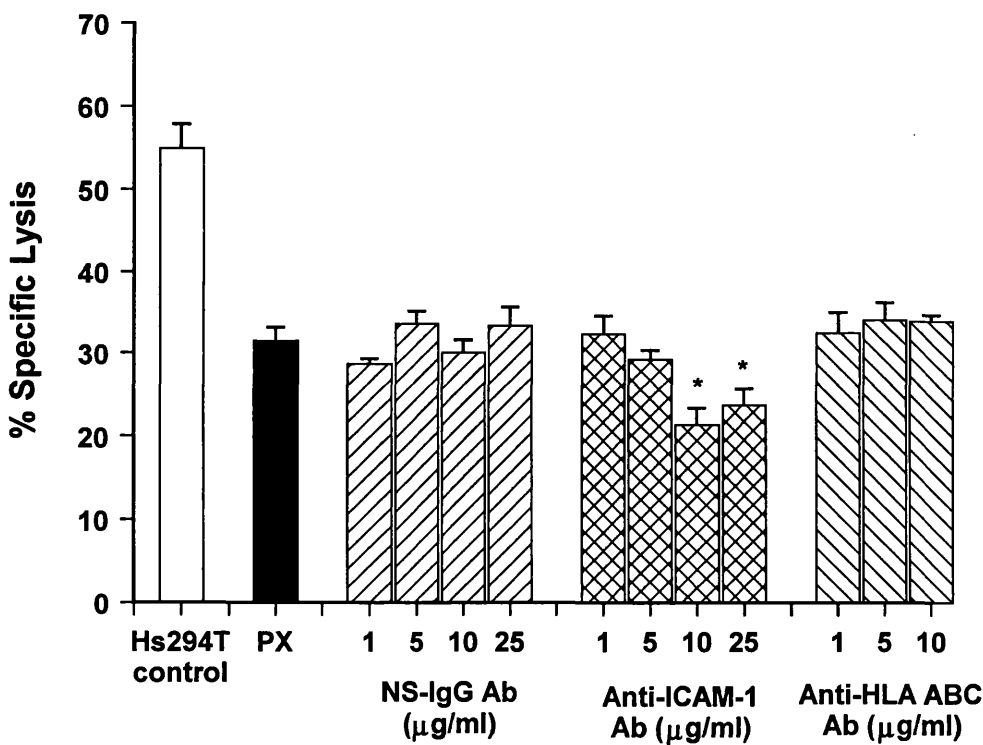
*Facs Analysis of mICAM-1 expression*

PX pretreatment over a range of concentrations (10-250µg/ml) was unable to significantly alter mICAM-1 expression (data not shown).



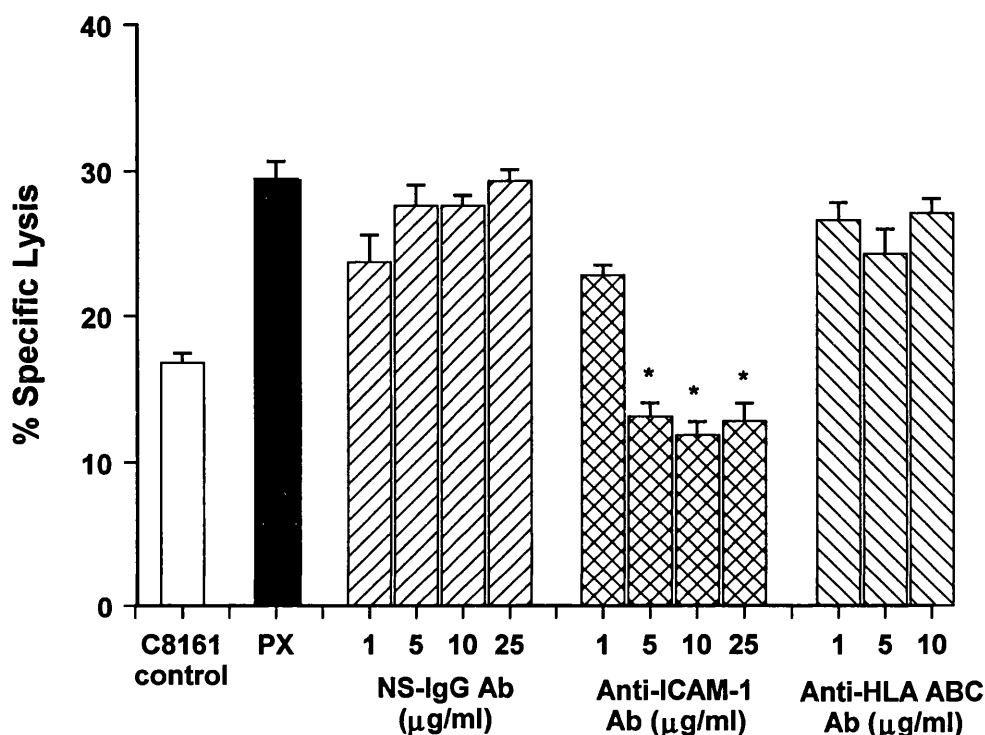
*Effect of anti-ICAM-1 and HLA-ABC antibody on LAK cell-mediated lysis of PX pretreated tumour cells.*

Both cell lines were pretreated for 4 days with 250µg/ml PX before each experiment. For all comparisons, lysis on addition of blocking antibody was compared to a non-specific IgG control antibody. 10µg/ml of blocking ICAM-1 antibody significantly reduced lysis of PX-pretreated Hs294T cells 1.5-fold from 30.0% to 21.3% ( $p<0.05$ ) and by 1.5-fold from 33.5% to 23.7% ( $p<0.05$ ) using 25µg/ml ICAM-1 antibody (Figure 27A). Blocking HLA-ABC antibody had no effect on LAK cell-mediated lysis of PX-pretreated Hs294T cells (Figure 27A).



**Figure 27A.** Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back hatched bar) on the lysis of Hs294T cells pretreated for 4 days with 250µg/ml PX (solid bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward hatched bar). Values are represented as the average  $\pm$  SEM where \* $P<0.05$  and represents comparison of control IgG with specific antibody.

In PX-pretreated C8161 cells, lysis was significantly reduced using 5µg/ml ICAM-1 antibody from 27.6% to 13.1% (2.1-fold,  $p<0.0005$ ) with similar decreases of 2.3-fold ( $p<0.0005$ ) using both 10µg/ml and 25µg/ml of blocking ICAM-1 antibody (Figure 27B). Blocking HLA-ABC antibody failed to alter LAK cell-mediated lysis of PX-pretreated C8161 cells.



**Figure 27B.** Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back hatched bar) on the lysis of C8161 cells pretreated for 4 days with 250µg/ml PX (solid bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward hatched bar). Values are represented as the average  $\pm$  SEM where \* $P<0.0005$  and represents comparison of control IgG with specific antibody.

Addition of blocking ICAM-1 antibody and blocking MHC class 1 antibody had no effect on lysis of PX-pretreated A375 cells (Figure 27C).

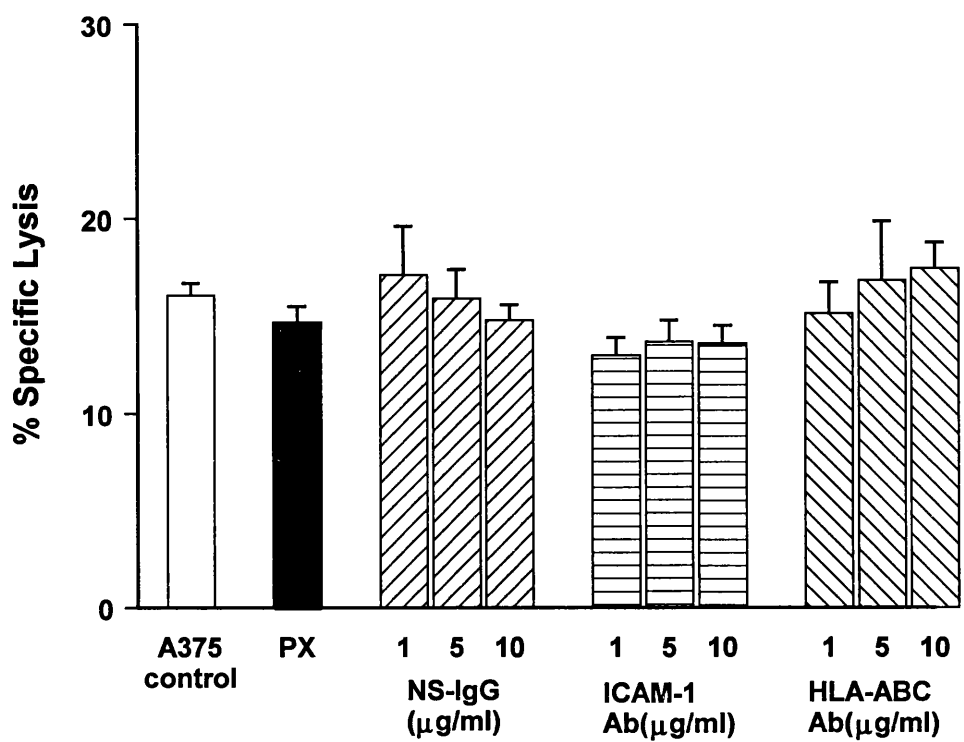
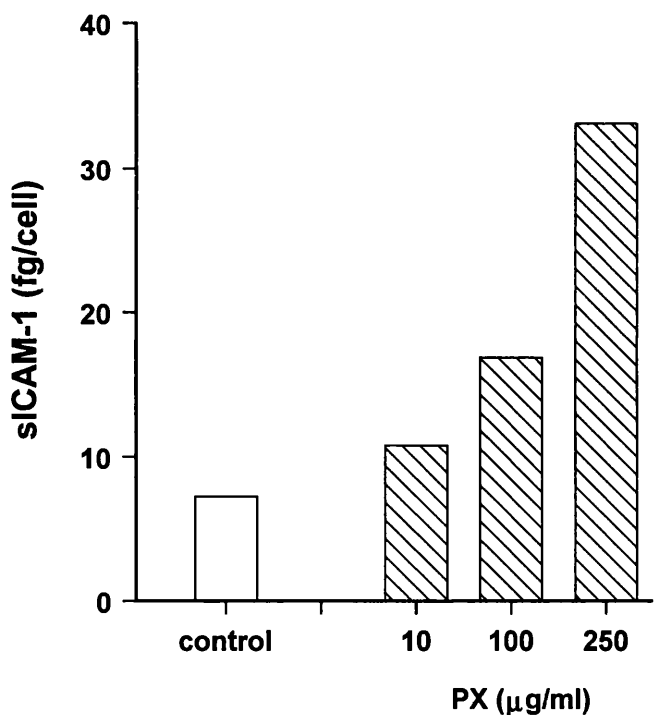


Figure 27C. Effect of blocking ICAM-1 antibody (cross-hatched bar) and blocking HLA-ABC antibody (back hatched bar) on the lysis of A375 cells pretreated for 4 days with 250µg/ml PX (solid bar) at an E:T ratio of 40:1. Non-specific IgG antibody was also included as a control (forward hatched bar). Values are represented as the average  $\pm$  SEM and represents comparison of control IgG with specific antibody.

**3.6. Effect of pentoxifylline on expression of sICAM-1**

The standard curve (Figure 24) was used to determine the amount of sICAM-1 released from untreated and PX-treated cell lines.

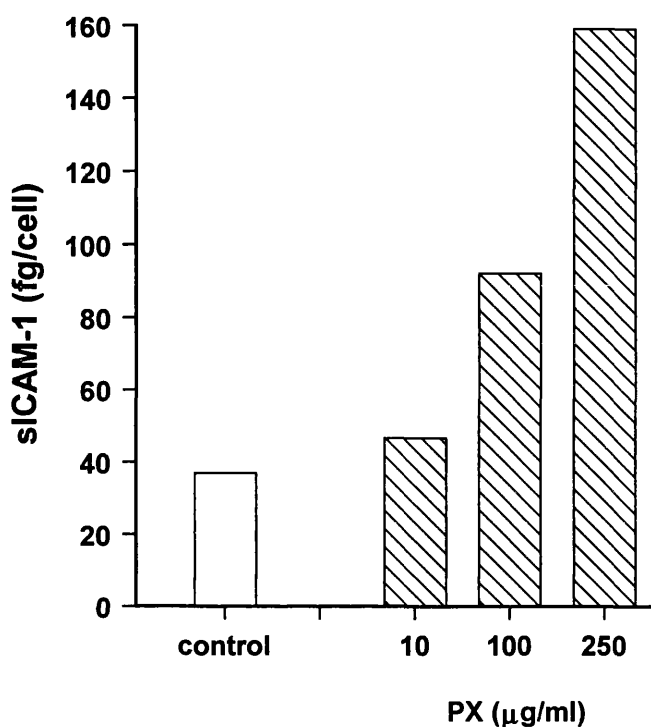
4 days pretreatment of the Hs294T cells with PX caused a dose dependent increase in sICAM-1 levels at all concentrations of drug tested (Figure 28A). Using 10µg/ml PX, sICAM-1 was increased 1.5-fold whereas, at the higher concentration of 100µg/ml PX, expression was increased 2.3-fold. A 4.7-fold increase was observed in the Hs294T cells on pretreatment of the tumour cells with 250µg/ml PX (Figure 28A).



**Figure 28A.** Effect of PX on sICAM-1 secretion from the Hs294T cells. Values represent the average of duplicate samples.

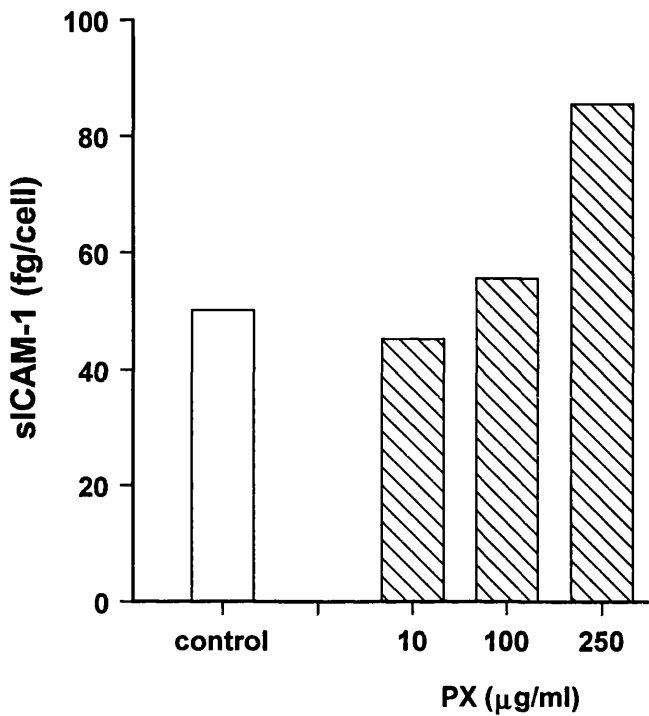
C8161 cells secreted basal levels of 36.9fg ICAM-1 per cell (Figure 28B).

Using 10µg/ml PX, sICAM-1 expression was increased 1.3-fold, and 2.5-fold using 100µg/ml PX. A 4.3-fold increase in sICAM-1 expression was observed on pretreatment of the tumour cells with 250µg/ml PX (Figure 28B).



**Figure 28B.** Effect of PX on sICAM-1 secretion from the C8161 cells. Values represent the average of duplicate samples.

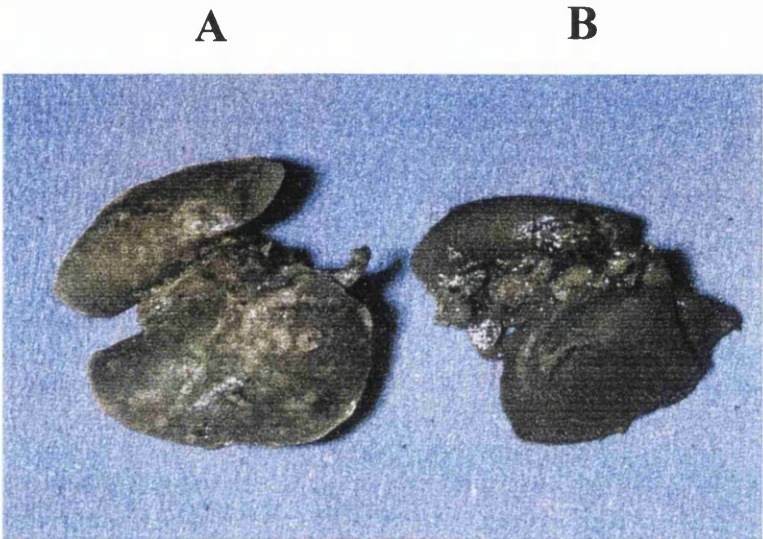
The A375 cells secreted basal levels of sICAM-1 of 50.5fg/cell (Figure 28C). Only at the higher concentration of PX (250µg/ml) was there an increase in sICAM-1 expression (from 50.5fg/cell to 85.5fg/cell).



**Figure 28C.** Effect of PX on sICAM-1 secretion from the A375 cells. Values represent the average of duplicate samples.

**3.7. Effect of retinoic acid and pentoxifylline on lung tumour colony formation using the experimental metastasis model**

The Hs294T cell line failed to produce any lung colonies and pretreatment of these cells with either 10<sup>-6</sup>M RA or 250µg/ml PX failed to alter tumour counts (data not shown). Both the A375 cell line and the C8161 cell line (Figure 29) produced lung tumour colonies (Tables 2A and 2B respectively), with the highly metastatic C8161 cells producing a much greater number of colonies (average of 31 compared to an average of 3 for the A375 cells).



**Figure 29.** Lung tumour colony formation using C8161 cells (A). Control lungs from a mouse not injected with tumour cells are shown in B.

Cell line	Treatment	No. lung colonies	Range	Average no. lung colonies
A375	control	0, 0, 0, 0, 0, 3, 8, 9	0-9	3
	10 <sup>-6</sup> M RA	0, 0, 0, 0, 0, 8, 61, 12	0-61	11
	250µg/ml PX	0, 0, 0, 3, 8, 4, 6, 4	0-5	2

**Table 2A.** Effect of RA and PX on A375 lung tumour colony formation.

Cell line	Treatment	No. lung colonies	Range	Average no. lung colonies
C8161	control	0, 3, 3, 19, 26, 88, 75	0-88	31
	10 <sup>-6</sup> M RA	0, 0, 0, 4, 14, 71, 103	0-103	28
	250µg/ml PX	0, 0, 6, 26, 42, 72, 100, 128	0-128	47

**Table 2B.** Effect of RA and PX on C8161 lung tumour colony formation.

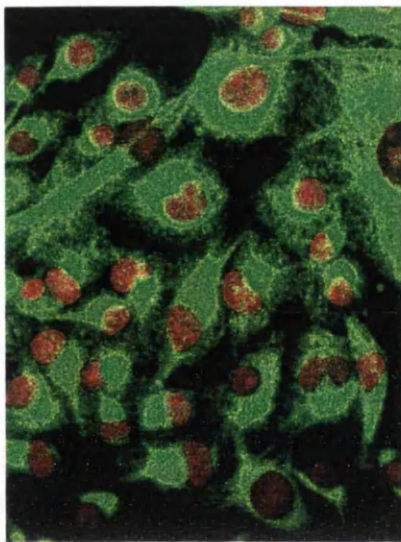
PX pretreatment of the A375 cell line appeared to have no significant effect on lung tumour formation (Table 2A), and although the average was greater for RA treatment compared to the control, this was due to the response of one mouse producing a much greater number of tumours than the rest of the group. The experiment would need to be repeated to see if this was a truly significant increase due to RA or simply an extreme response by one mouse.

10<sup>-6</sup>M RA pretreatment of the C8161 cells did not produce any striking effect on lung tumour formation (Table 2B). Although PX produced a greater number of tumours on average than the untreated A375 cells, again, due to the variation in responses of the mice, it is difficult to conclude this difference is due to the drug treatment as most of the lung tumour counts were similar in both groups with only a few exceptions. The experiment would have to be repeated on a much larger scale to provide sound evidence that either drug had any effect on tumour colony formation.

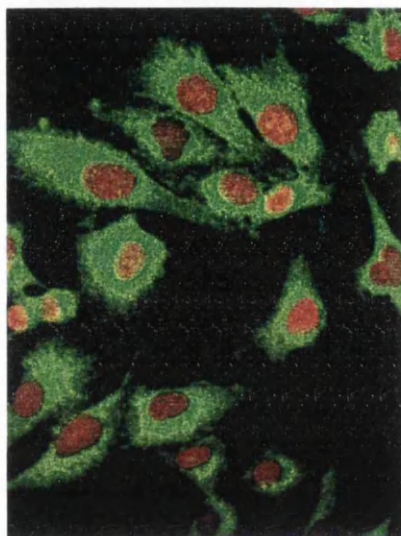


### *Confocal microscopy of membrane-associate uPA and tPA expression*

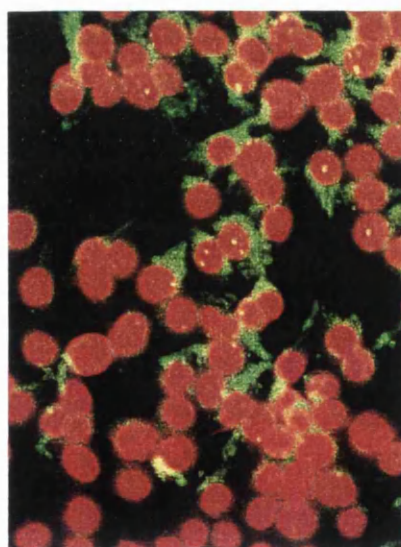
Cell-associated uPA was strongly expressed in greater than 95% of C8161 cells (Figure 30A) and Hs294T cells (Figure 30B), and although the A375 cells did express uPA (Figure 30C), expression was much weaker than the other two cell lines. tPA was also found to be associated with greater than 95% of the C8161, Hs294T and A375 cells (Figures 31A, 31B and 31C respectively) although at much lower levels than uPA. Controls staining with secondary antibody only were negative (Figures 32A, B, C).



A

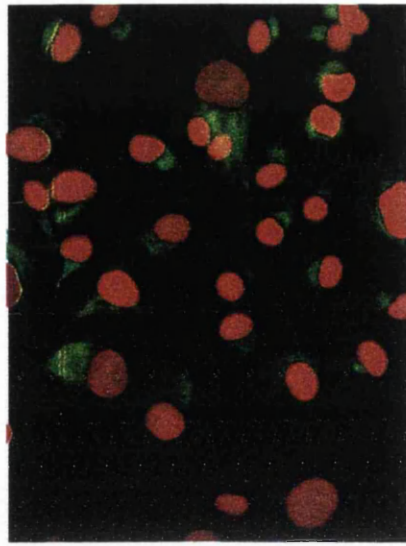


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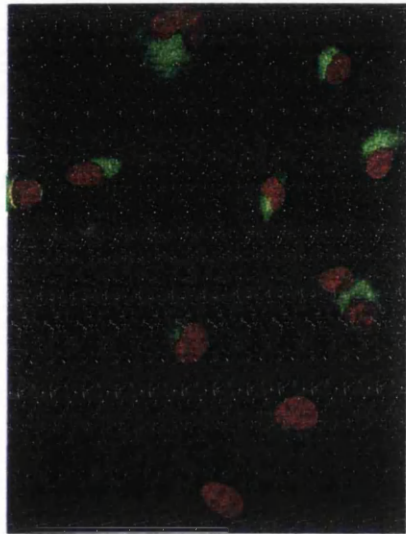


C

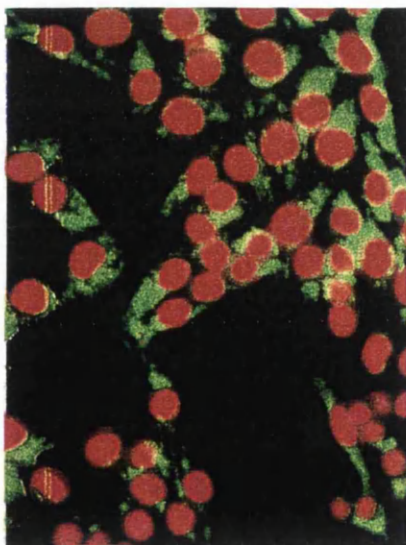
**Figure 30.** Expression of uPA in A) C8161 cells; B) Hs294T cells; C) A375 cells.



A

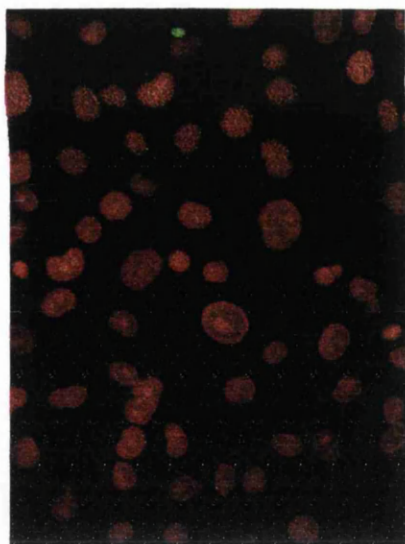


B

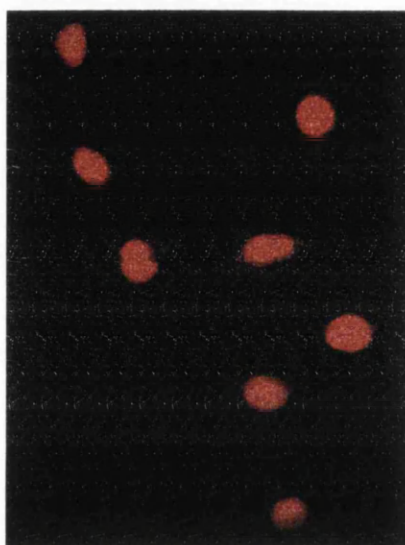


C

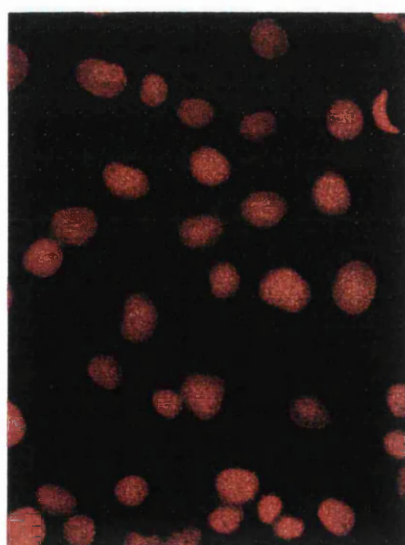
**Figure 31.** Expression of tPA in A) C8161 cells; B) Hs294T cells; C) A375 cells.



A



B

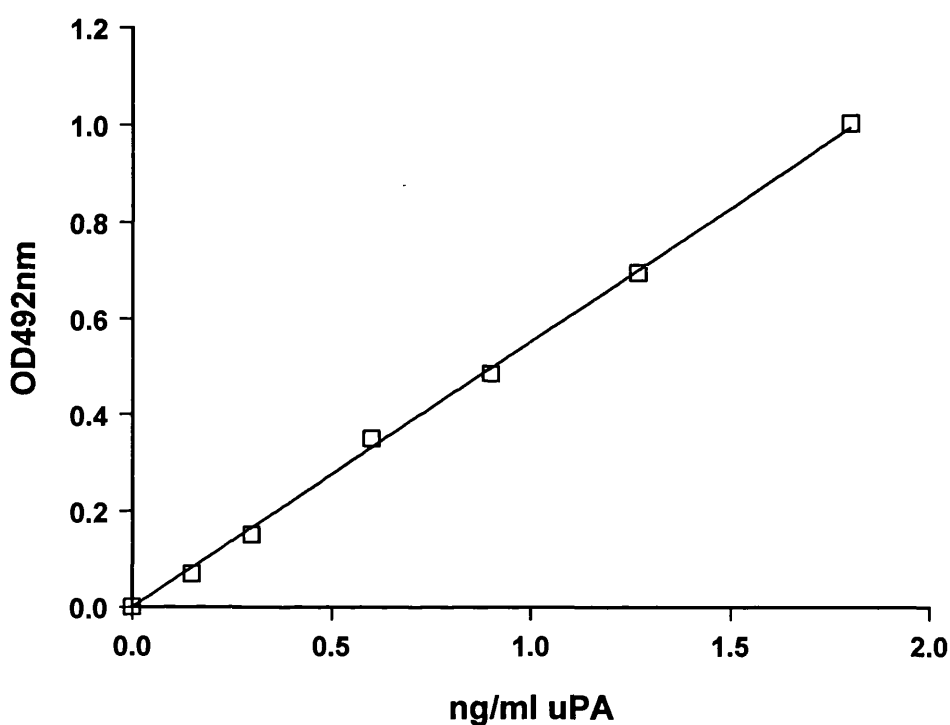


C

**Figure 32.** Staining of A) C8161 cells; B) Hs294T cells; C) A375 cells with secondary FITC-conjugated antibody used in uPA and tPA staining.

### **3.8. ELISA determination of secreted uPA and tPA**

For each experiment, standards of known concentration of either uPA or tPA were used to plot a standard curve. The equation of the line was calculated for each experiment and used to allow the concentration of PA from the cell lines to be determined. The initial standard curve measuring uPA levels (Figure 33) allowed samples of unknown concentrations of uPA with optical density values no greater than 1 to be determined.



**Figure 33.** Standard curve of uPA where  $y=0.552x$ . All samples were performed in duplicate and are represented as the average.

*Preliminary experiments to determine the range of dilutions required to measure secreted uPA in the A375, C8161 and Hs294T cells.*

Initially, the supernatants from untreated Hs294T, C8161 and A375 cells were added to the ELISA plates over a range of dilutions from 1/5 to 1/200. The unknown concentration of uPA in each cell line was determined from figure 33, and the results expressed as the amount of uPA secreted per cell (Table 3).

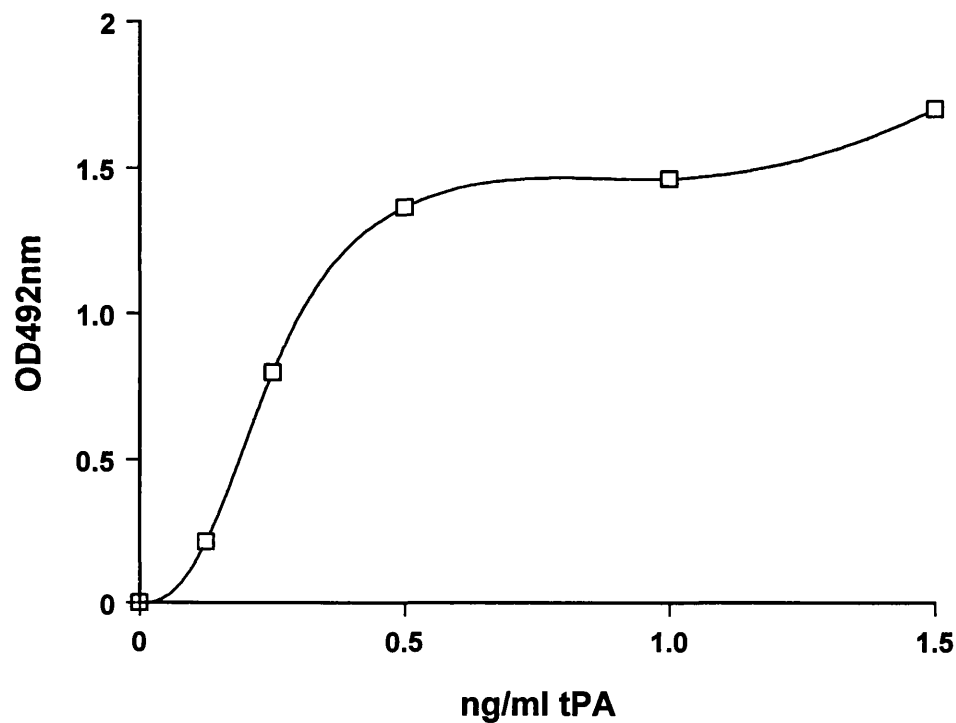
<b>Hs294T Dilution</b>	<b>Absorbance (ng/ml)</b>	<b>uPA (ng/5ml)</b>	<b>total uPA (fg/cell)</b>	<b>uPA/cell</b>
undiluted	0.301	0.55	2.75	2.37
1/5	0.052	0.47	2.35	2.02
1/20	0.035	1.27	6.35	5.47
1/50	0.0195	1.35	6.75	5.81
1/100	0.0075	1.77	8.85	7.62
1/200	0	0	0	0
1/400	0	0	0	0

**Table 3.** Initial measurement of uPA secretion from the Hs294T cell line. Samples were analysed in duplicate and the amount of uPA determined by standards in figure 33.

In preliminary experiments, over the range of chosen dilutions, uPA expression was undetectable in the A375 cell line, whereas, in contrast, using the C8161 cell line, the absorbancies obtained were outwith the limits of the uPA assay indicating the C8161 supernatant contained levels of uPA which were too high to be measured even at the highest dilution (data not shown). In the Hs294T cell line, uPA expression was detectable at the selected dilutions and increased with increasing dilution factor (Table 3) from 2.4fg/cell in undiluted supernatant to 7.6fg/cell at a dilution factor of 1/100. At the higher dilutions of 1/200 and 1/400, the uPA in the sample became so dilute that it was undetectable.

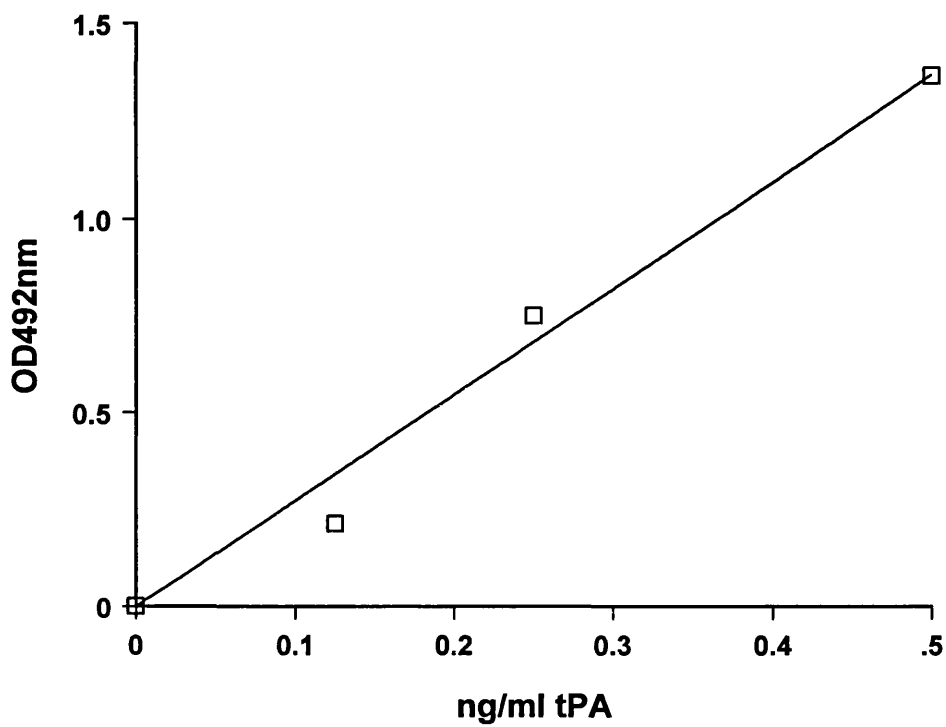
*Preliminary measurements of tPA expression*

The standard curve produced from samples of known concentrations of tPA produced a plateau rather than a straight line (Figure 34A).



**Figure 34A.** Standard curve of tPA.

Therefore, the linear part of figure 34A was used to construct the standard curve (Figure 34B) to determine the concentration of tPA in the cell line supernatants.



**Figure 34B.** Linear part of figure PA2 used to determine the amount of secreted tPA from the tumour cell lines where  $y=2.73x$ .



Using Figure 34B, the expression of tPA from all three cell lines was determined (Table 4).

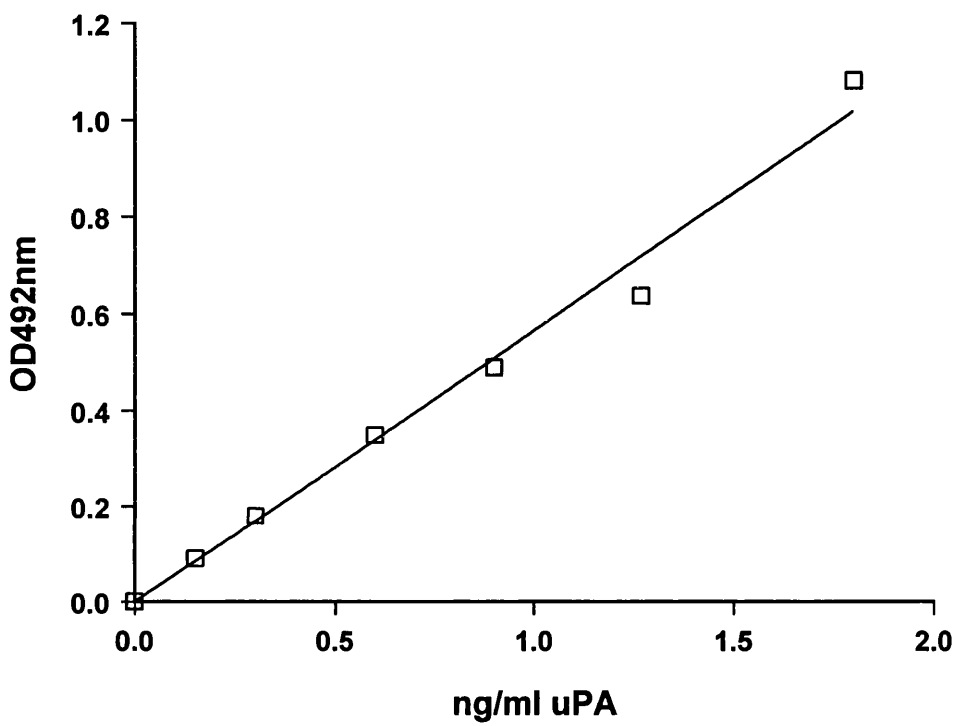
Cell line	Expression of tPA (fg/cell)					
	Undiluted	1/5	1/20	1/50	1/100	1/200
C8161	0.66	1.04	1.16	1.60	1.60	2.31
Hs294T	*	7.72	9.59	11.23	12.14	15.77
A375	*	7.15	10.69	13.05	12.35	11.38

**Table 4.** Expression of tPA from untreated C8161, Hs294T and A375 cells. The data represents the average of duplicate samples. \* denotes samples with absorbance values outwith the valid range of the assay.

All three tumour cell lines were found to express tPA (Table 4) with the C8161 cells secreting the lowest levels (range 0.7 - 2.3fg/cell) over the range of dilutions tested. Both the the Hs294T cells (7.7 - 15.8fg/cell) and the A375 cells (7.2 - 13.1fg/cell) secreted higher levels of tPA, with expression of tPA from either cell line being similar. As with the initial determination of uPA expression, the general trend appeared to show that levels of tPA increased as the samples became more dilute (Table 4).

**3.9. Retinoid modulation of uPA expression**

A fresh batch of cells were cultured with and without 4 days pretreatment using a range of concentrations of RA ( $10^{-10}\text{M}$  -  $10^{-5}\text{M}$ ) and the supernatant collected to determine uPA expression. Figure 35 was constructed to allow the expression of uPA to be calculated from the samples.



**Figure 35.** Standard curve of uPA where  $y=0.565x$ . Data represents the average of duplicate samples.

RA was unable to induce expression of uPA from the A375 cells which, in the preliminary experiment, had been shown to lack expression of uPA (data not shown).

Using the C8161 cells, absorbancies from the supernatants diluted 1/600 (Table 5) from cells pretreated with  $10^{-7}\text{M}$  -  $10^{-5}\text{M}$  RA were outwith the valid range of the assay (as determined by Figure 35). However, at the 1/800 dilution, RA caused a maximum 2.4-fold increase in uPA expression in the C8161 cell line using  $10^{-7}\text{M}$  RA (Table 5).

C8161	Control	$10^{-10}\text{M}$ RA	$10^{-9}\text{M}$ RA	$10^{-8}\text{M}$ RA	$10^{-7}\text{M}$ RA	$10^{-6}\text{M}$ RA	$10^{-5}\text{M}$ RA
1/600	1.06	1.30	1.38	2.47	*	*	*
1/800	1.21	1.05	1.2	2.13	2.86	2.72	2.57

**Table 5.** Determining the amount of secreted uPA from the C8161 cell line with and without 4 days pretreatment with a range of concentrations of RA. Figure 35 was used to determine uPA expression and \* denotes samples whose absorbances were outwith the valid range of the assay. All samples were performed in duplicate and values expressed as pg uPA/cell. All values at the 1/400 dilution were outwith the valid range of the assay.

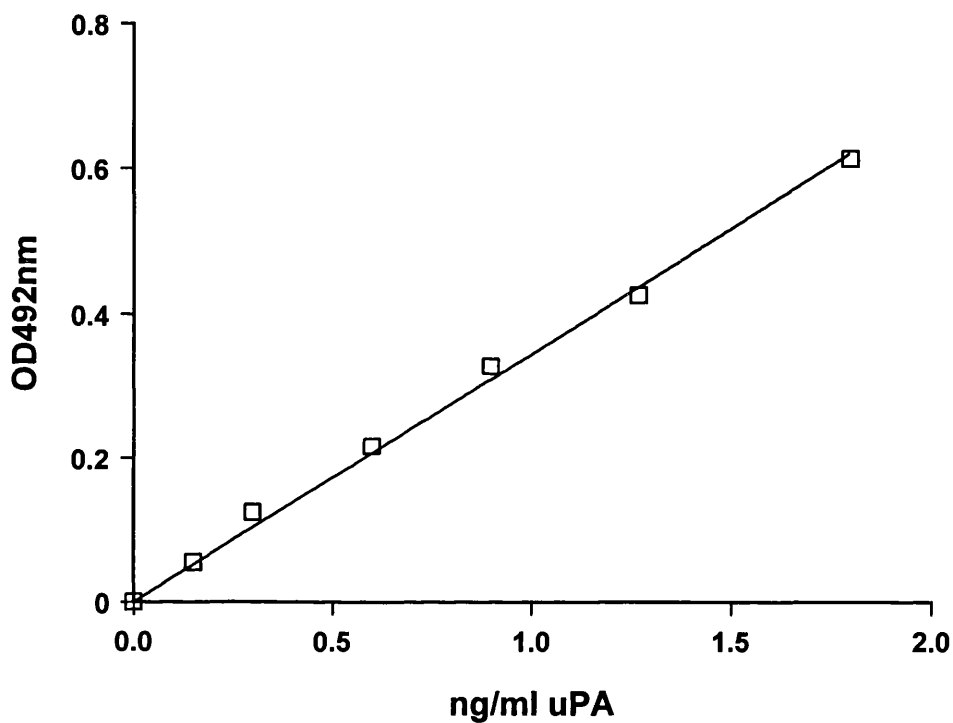
Using the Hs294T cells, there was a slight increase in uPA secretion on treatment of the tumour cells with RA, with a maximum 2-fold increase observed using  $10^{-9}\text{M}$  RA (1/20 and 1/50 dilution) (Table 6).

Hs294T	Control	$10^{-10}\text{M}$ RA	$10^{-9}\text{M}$ RA	$10^{-8}\text{M}$ RA	$10^{-7}\text{M}$ RA	$10^{-6}\text{M}$ RA	$10^{-5}\text{M}$ RA
1/5	1.79	1.68	1.38	1.92	3.68	4.24	3.07
1/20	3.14	1.52	6.55	2.61	3.89	3.29	4.49
1/50	4.66	2.44	8.98	2.62	5.67	3.65	2.93

**Table 6.** Determining the amount of secreted uPA from the Hs294T cell line with and without 4 days pretreatment with a range of concentrations of RA. Figure 35 was used to determine uPA expression and all samples were performed in duplicate with values expressed as fg uPA/cell.

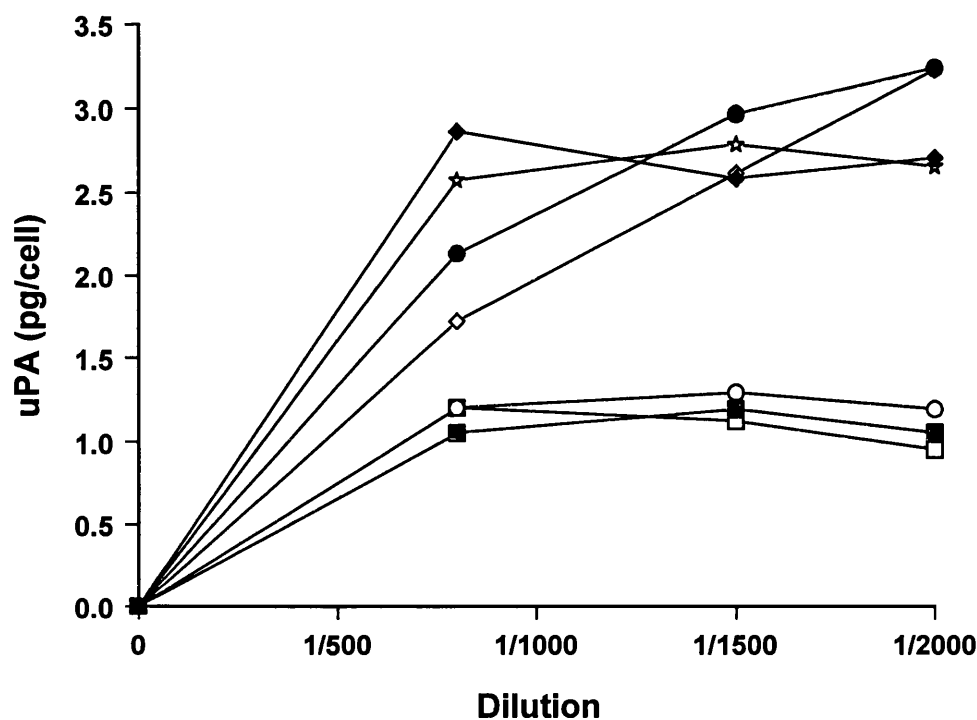
**3.9.1 Repeating the experiments on a new batch of tumour cells to examine the retinoid modulation of uPA expression**

A new batch of tumour cells were set up with and without 4 days pretreatment with RA ( $10^{-10}\text{M}$  -  $10^{-5}\text{M}$ ). The standard curve was constructed for uPA using the standards (Figure 36) to allow determination of expression of uPA from the experimental samples.



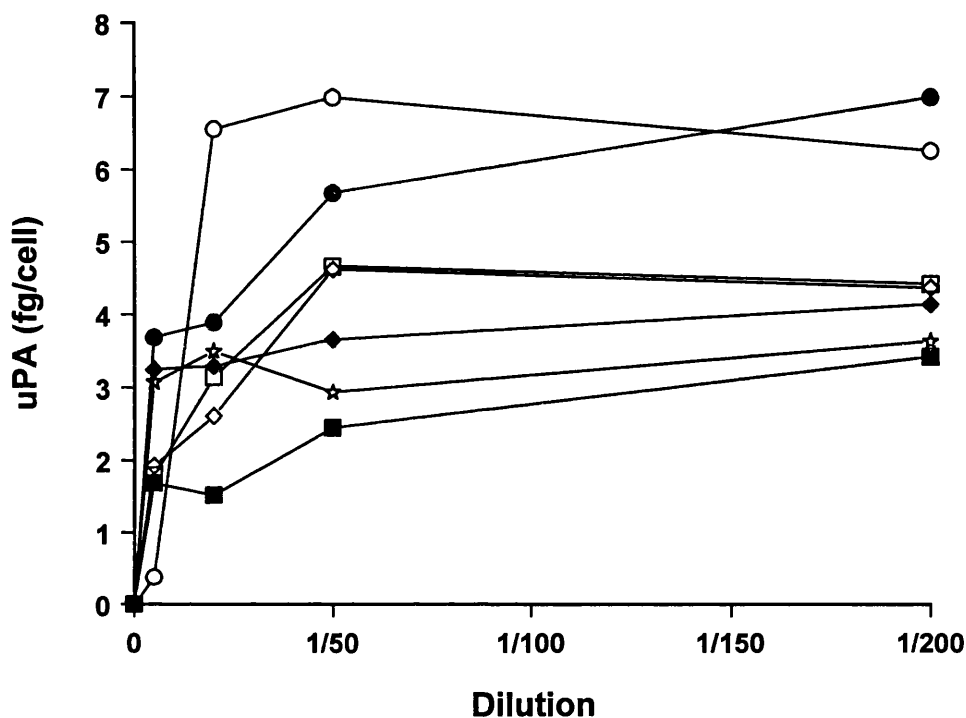
**Figure 36.** Standard curve of uPA where  $y=0.344x$ .

Figure 37A shows that RA ( $10^{-8}\text{M}$  -  $10^{-5}\text{M}$ ) induced an increase in uPA expression with the lower concentrations of  $10^{-10}\text{M}$  -  $10^{-9}\text{M}$  having minimal effects on uPA expression. A maximum value of 3230fg uPA/cell was achieved using  $10^{-8}\text{M}$  RA which corresponded to a 3.4-fold increase in uPA expression at the 1/2000 dilution.



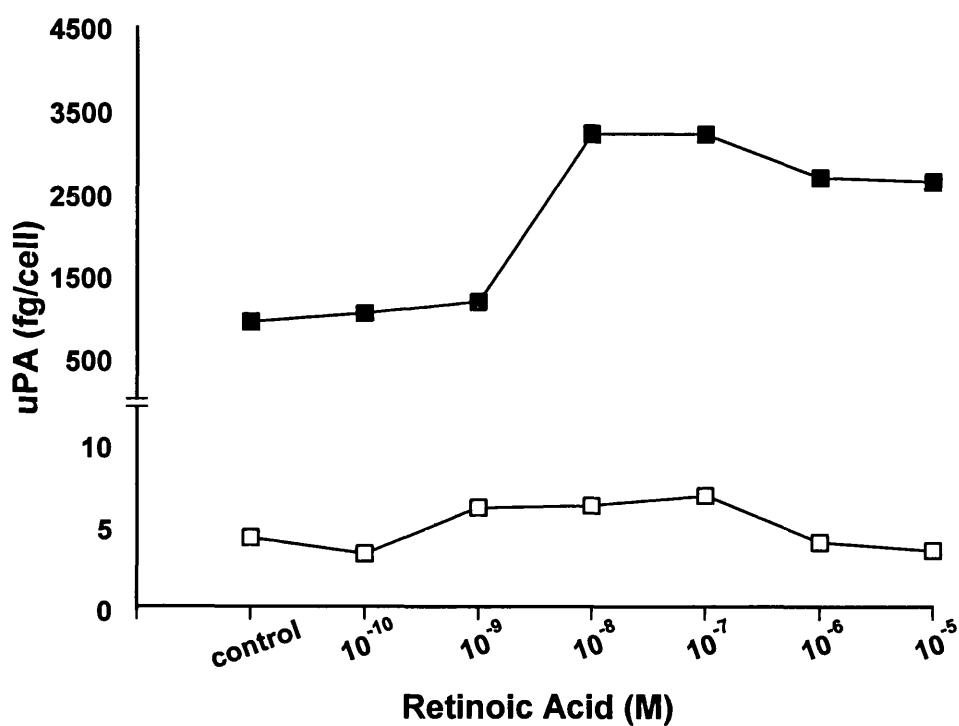
**Figure 37A.** Effect of RA (■ $10^{-10}\text{M}$ ; ○ $10^{-9}\text{M}$ ; ● $10^{-8}\text{M}$ ; ◇ $10^{-7}\text{M}$ ; ◆ $10^{-6}\text{M}$ ; ☆ $10^{-5}\text{M}$ ; □control) on secretion of uPA from the C8161 cell line. Data represents the average of duplicate samples where the secretion of uPA from the unknowns were calculated from the standards from figure 36. Absorbance readings at the dilution of 1/4000 were negative for all samples tested.

10<sup>-9</sup>M RA and 10<sup>-8</sup>M RA induced an increase in uPA expression in the Hs294T cell line, with maximum expression achieved using 10<sup>-8</sup>M RA which increased uPA expression from 4.41fg/cell to 7.0fg/cell at the 1/200 dilution (Figure 37B). Levels of expression decreased to similar values as the control using 10<sup>-6</sup>M and 10<sup>-5</sup>M RA.



**Figure 37B.** Effect of RA (■10<sup>-10</sup>M; ○10<sup>-9</sup>M; ●10<sup>-8</sup>M; ◇10<sup>-7</sup>M; ◆10<sup>-6</sup>M; ☆10<sup>-5</sup>M; □control) on secretion of uPA from the Hs294T cell line. Data represents the average of duplicate samples where the secretion of uPA from the unknowns were calculated from the standards from figure 36. Absorbance readings at the dilution of 1/400 were negative for all samples tested.

Figure 38 combines the data from figures 37A and 37B at the highest dilution tested for each cell line (1/2000 for the C8161 cells and 1/200 for the Hs294T cells) to show a comparison between the retinoid-induced alterations in uPA expression in the 2 cell lines.

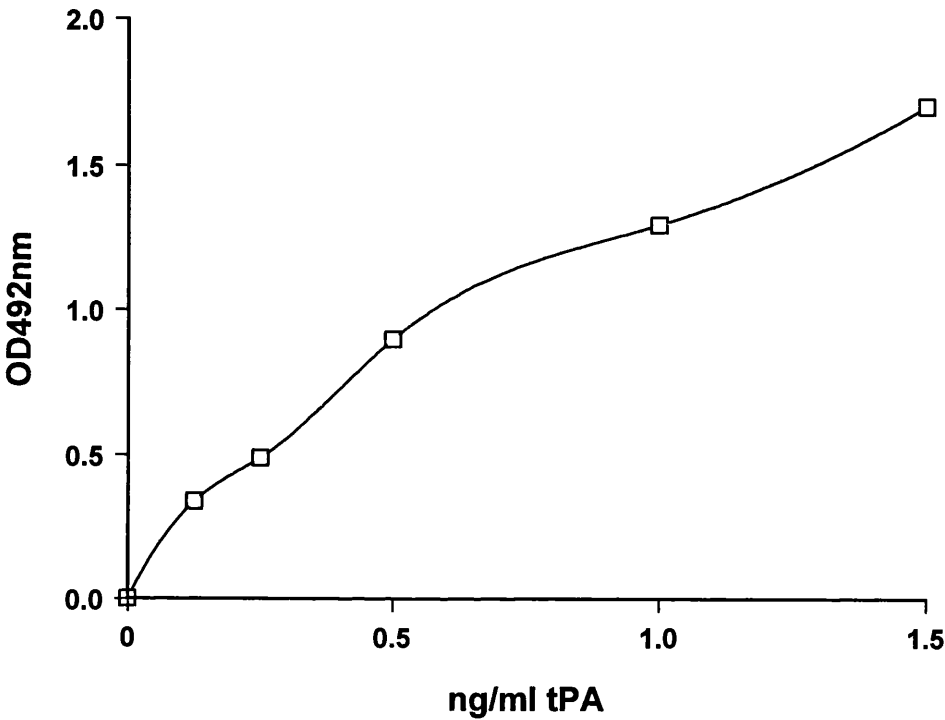


**Figure 38.** Comparison of uPA secretion from C8161 cells (■) and Hs294T cells (□). Values represent the average of duplicate samples.

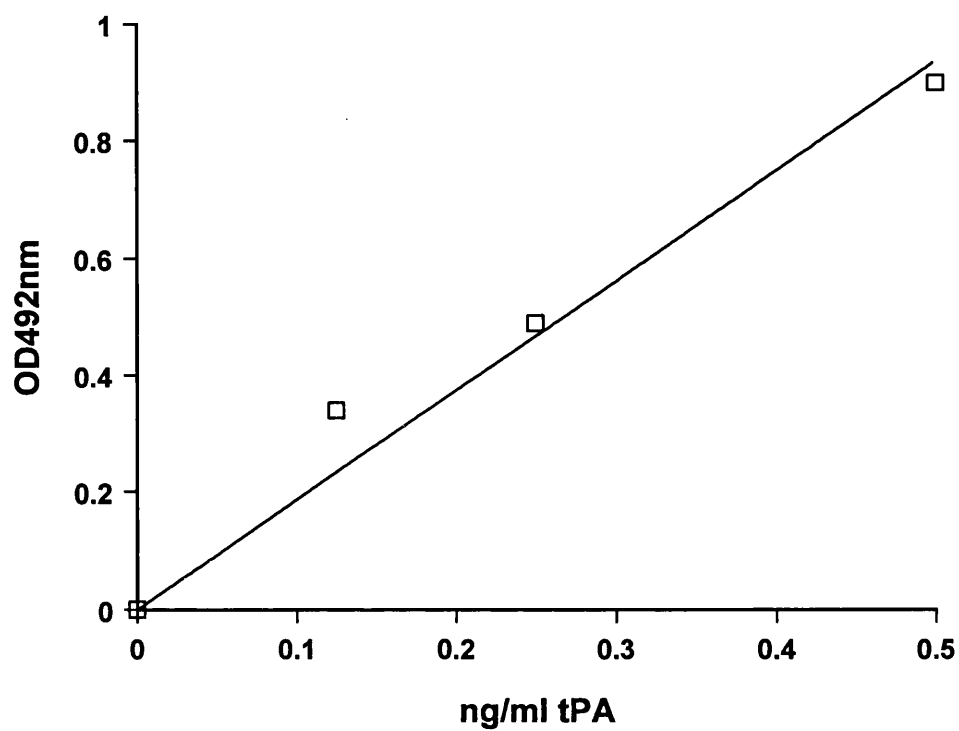


**3.10. Retinoid modulation of tPA expression**

Figure 39A was constructed to allow the unknown concentrations of tPA to be determined from the same supernatant used to analyse uPA expression (Tables 5 and 6). As with the previous tPA assay, the linear part of the graph was used to determine tPA levels in the experimental samples (Figure 39B).



**Figure 39A.** Standard curve of tPA.



**Figure 39B.** Linear part of figure 39B used to determine the amount of secreted tPA from the tumour cell lines where  $y=1.867x$ . Values represent the average of duplicate samples.

Expression of tPA from the C8161 cells was shown to increase as the dilution factor increased (Table 7). At the 1/50 dilution,  $10^{-6}$ M RA induced a 3.9-fold increase in tPA expression, with maximum expression induced (5.6-fold) using  $10^{-5}$ M RA. Similar increases were found at the 1/200 dilution where  $10^{-6}$ M RA induced a 3.1-fold increase in tPA expression compared to a 3.8-fold increase using  $10^{-5}$ M RA (Table 7).

C8161	Control	$10^{-10}$ M RA	$10^{-9}$ M RA	$10^{-8}$ M RA	$10^{-7}$ M RA	$10^{-6}$ M RA	$10^{-5}$ M RA
1/5	1.61	2.45	2.3	2.48	*	*	*
1/50	2.24	2.89	3.76	3.92	6.64	8.64	12.63
1/200	4.29	3.5	7.58	9.22	9.51	13.3	16.12

**Table 7.** Determining the amount of secreted tPA from the C8161 cell line with and without 4 days pretreatment with a range of concentrations of RA. Figure 39B was used to determine tPA expression and \* denotes samples whose absorbances were outwith the valid range of the assay. All samples were performed in duplicate and values expressed as fg tPA/cell.

The expression of tPA also increased in the Hs294T cells with increasing dilution factor (Table 8). At the 1/50 dilution, maximum induction of tPA expression was observed with 10<sup>-7</sup>M RA, which increased tPA levels 2.5-fold. Expression was increased 4.6-fold with the same pretreatment at the higher dilution of 1/200 (Table 8).

Hs294T	Control	10 <sup>-10</sup> M RA	10 <sup>-9</sup> M RA	10 <sup>-8</sup> M RA	10 <sup>-7</sup> M RA	10 <sup>-6</sup> M RA	10 <sup>-5</sup> M RA
1/50	25.62	19.58	37.21	40.42	64.25	41.29	38.94
1/200	21.02	24.39	79.79	88.85	97.16	81.50	79.63

**Table 8.** Determining the amount of secreted tPA from the Hs294T cell line with and without 4 days pretreatment with a range of concentrations of RA. Figure 39B was used to determine tPA expression. All samples were performed in duplicate and values expressed as fg tPA/cell. Absorbances for all samples diluted 1/5 were outwith the valid range of the assay.

As was shown for the Hs294T and C8161 cells, expression of tPA from the A375 cells was increased in the more dilute sample (Table 9). Pretreatment of the tumour cells with 10<sup>-7</sup>M RA caused a 1.7-fold increase in tPA secretion which was similar using 10<sup>-6</sup>M RA (Table 9).

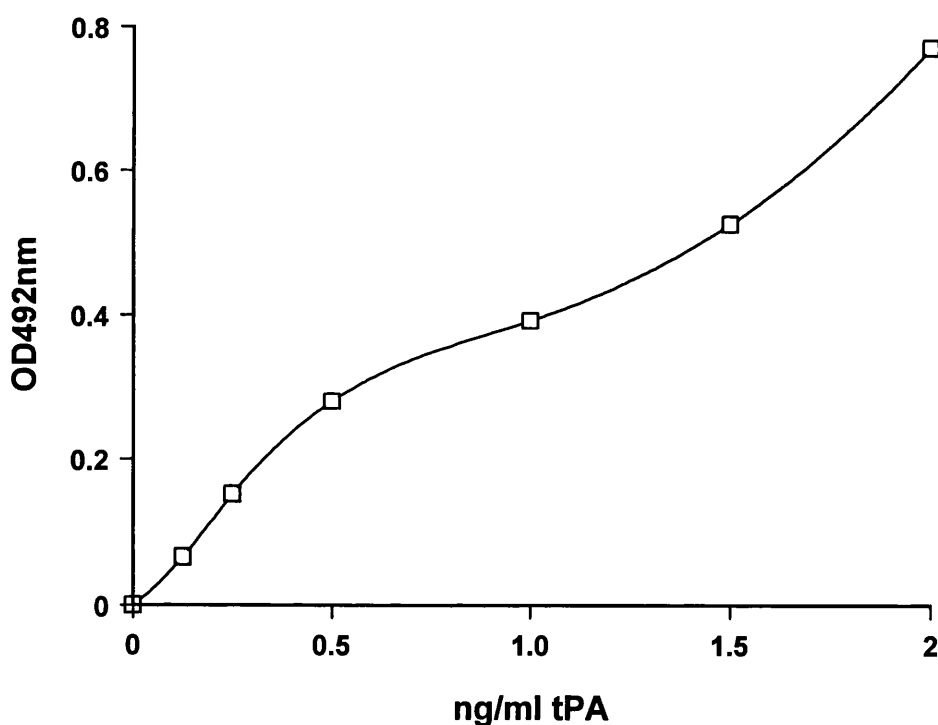
A375	Control	10 <sup>-10</sup> M RA	10 <sup>-9</sup> M RA	10 <sup>-8</sup> M RA	10 <sup>-7</sup> M RA	10 <sup>-6</sup> M RA	10 <sup>-5</sup> M RA
1/50	15.01	11.39	17.43	16.73	27.89	26.63	23.36
1/200	27.98	23.10	27.05	32.72	46.82	48.62	33.31

**Table 9.** Determining the amount of secreted tPA from the A375 cell line with and without 4 days pretreatment with a range of concentrations of RA. Figure 39B was used to determine tPA expression. All samples were performed in duplicate and values expressed as fg tPA/cell. Absorbances for all samples diluted 1/5 were outwith the valid range of the assay and readings at the 1/400 dilution were zero.

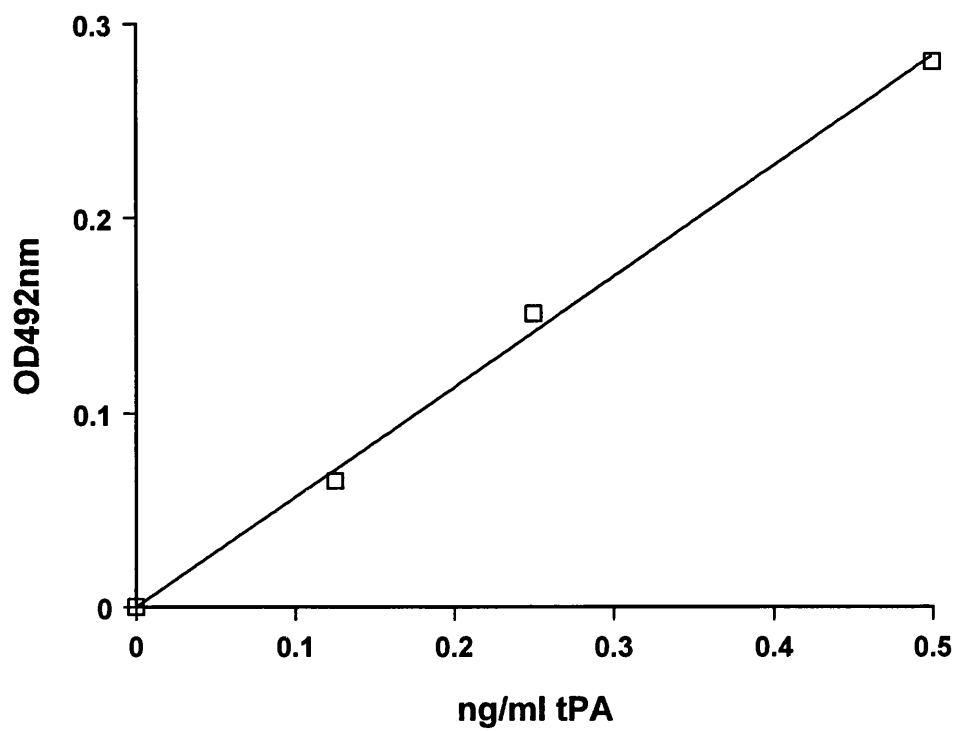
### **3.10.1. Repeating the experiments on a fresh batch of cells to determine the retinoid-induced modulation of tPA expression**

It was not possible to repeat all the tPA assays due to the difficulty in obtaining more chicken anti-human tPA antibody. Therefore, since the maximum values of tPA expression were obtained with the RA-pretreated A375 cells at the chosen dilutions, it was decided to repeat the experiments with the Hs294T and C8161 cells at higher dilutions to obtain values for the maximum expression of secreted tPA with RA treatment.

Using the same supernatant as used to produce the data in figures 37A, B and 38, tPA expression was determined in the samples by first constructing a standard curve using known standards (Figures 40A and 40B).

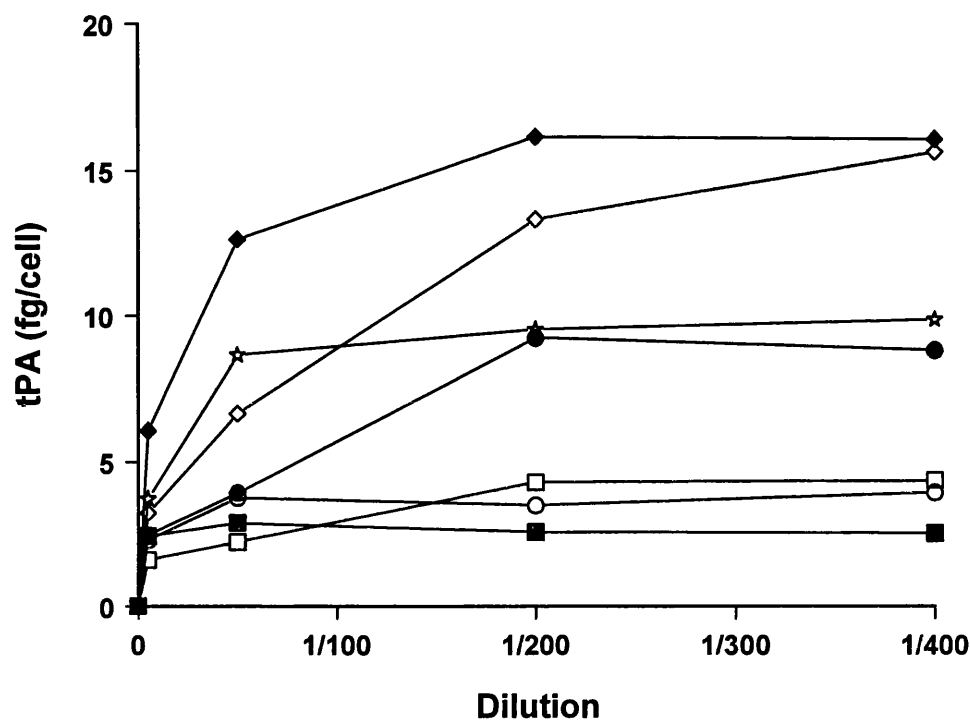


**Figure 40A.** Standard curve of tPA.



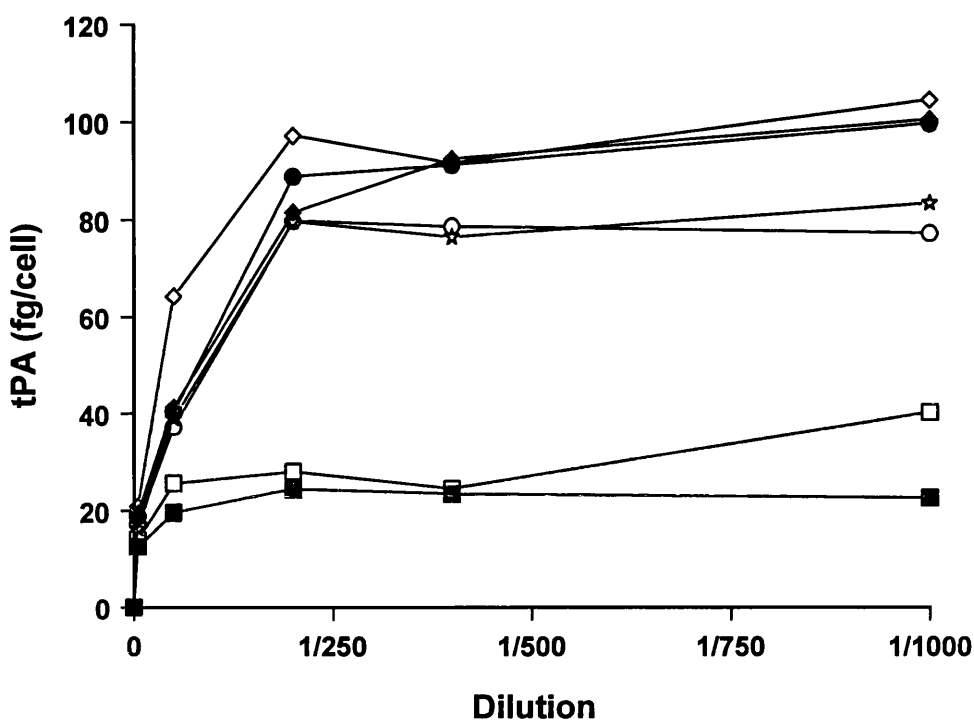
**Figure 40B.** Linear part of figure 40A used to determine the amount of secreted tPA from the tumour cell lines where  $y=0.566x$ .

Expression of tPA was increased with  $10^{-8}\text{M}$  -  $10^{-5}\text{M}$  RA pretreatment of the C8161 cells (Figure 41A). Maximum expression was reached using  $10^{-6}\text{M}$  RA which increased expression from 4.34fg/cell to 16fg/cell at the 1/400 dilution.



**Figure 41A.** Effect of RA (■ $10^{-10}\text{M}$ ; ○ $10^{-9}\text{M}$ ; ● $10^{-8}\text{M}$ ; ◇ $10^{-7}\text{M}$ ; ◆ $10^{-6}\text{M}$ ; ☆ $10^{-5}\text{M}$ ; □control) on secretion of tPA from C8161 cells . Data represents the average of duplicate samples where the secretion of tPA from the unknowns were calculated from the standards from Figure 40B. Absorbance readings at the dilution of 1/800 were negative for all samples tested.

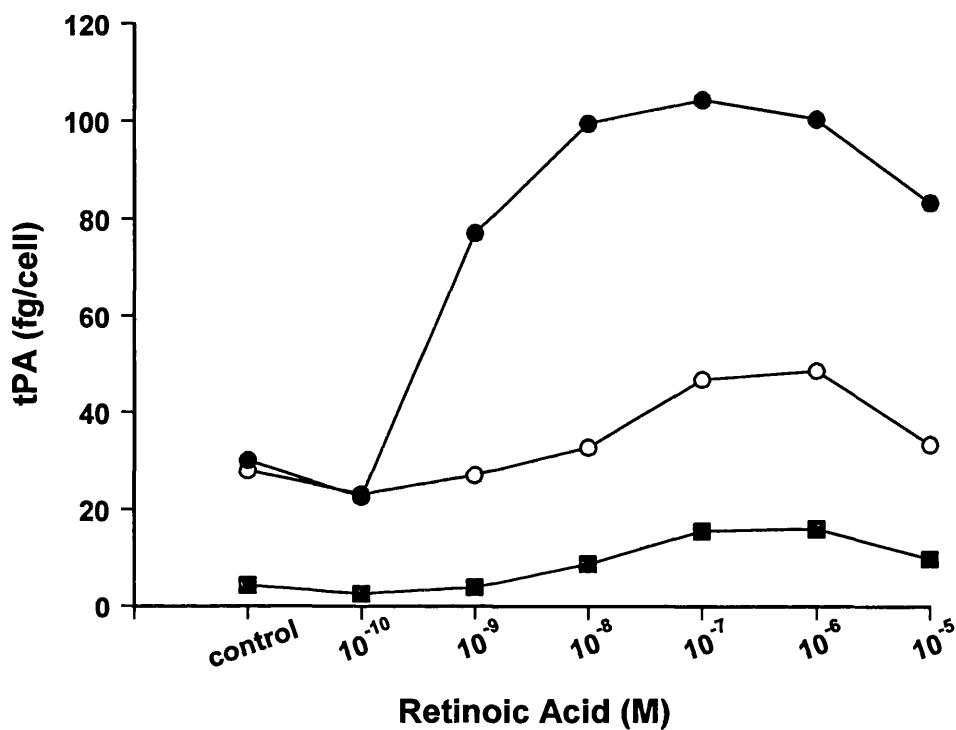
tPA expression was also induced in the Hs294T cell line using RA at concentrations of  $10^{-9}\text{M}$  -  $10^{-5}\text{M}$  (Figure 41B). At the 1/400 dilution expression was increased from 24.5fg/cell to 92.4fg/cell using  $10^{-6}\text{M}$  RA.



**Figure 41B.** Effect of RA (■ $10^{-10}\text{M}$ ; ○ $10^{-9}\text{M}$ ; ● $10^{-8}\text{M}$ ; ◇ $10^{-7}\text{M}$ ; ◆ $10^{-6}\text{M}$ ; ☆ $10^{-5}\text{M}$ ; □control) on secretion of tPA from Hs294T cells. Data represents the average of duplicate samples where the secretion of tPA from the unknowns were calculated from the standards from Figure 40B. Absorbance readings at the dilution of 1/4000 were negative for all samples tested.



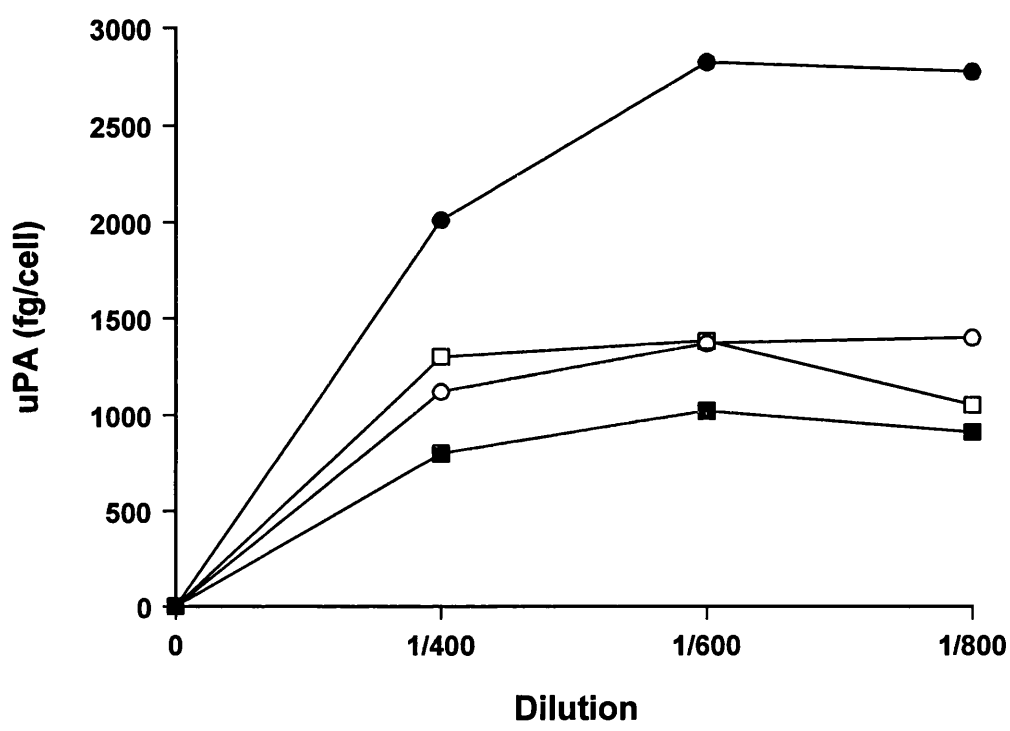
Figure 42 combines the data from Figures 41A and 41B and Table 9 at the highest dilution tested for each cell line (1/400 for the C8161 cells; 1/1000 for the Hs294T cells and 1/200 for the A375 cells) to show a comparison between the retinoid-induced alterations in tPA expression in the 3 cell lines.



**Figure 42.** Comparison of tPA secretion from C8161 cells (■); A375 cells (○) and Hs294T cells (●). Values represent the average of duplicate samples.

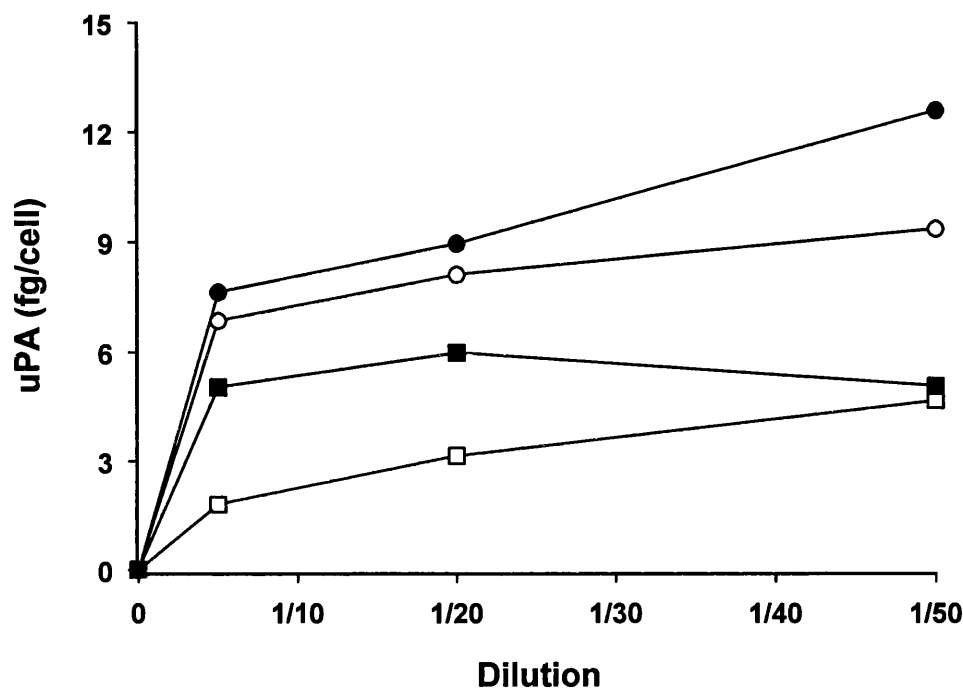
**3.11. Pentoxifylline modulation of uPA expression**

The ELISA assays analysing the effect of PX on uPA expression have only been performed once, and would need to be repeated before any final conclusions could be drawn. Using the C8161 cells, the lower concentrations of PX fail to significantly modulate uPA expression. However, using 250µg/ml PX, expression of uPA was greatly increased (2.6-fold at the 1/800 dilution) (Figure 43A).



**Figure 43A.** Effect of PX (■10µg/ml; ○100µg/ml; ●250µg/ml; □control) on secretion of uPA from the C8161 cell line. Figure 35 was used to determine uPA expression and all samples were performed in duplicate with values expressed as fg uPA/cell.

The general trend appears to show that PX induces an increase in uPA secretion from the Hs294T cells with a maximum 2.7-fold increase being achieved after 4 days pretreatment with 250µg/ml PX (Figure 43B).

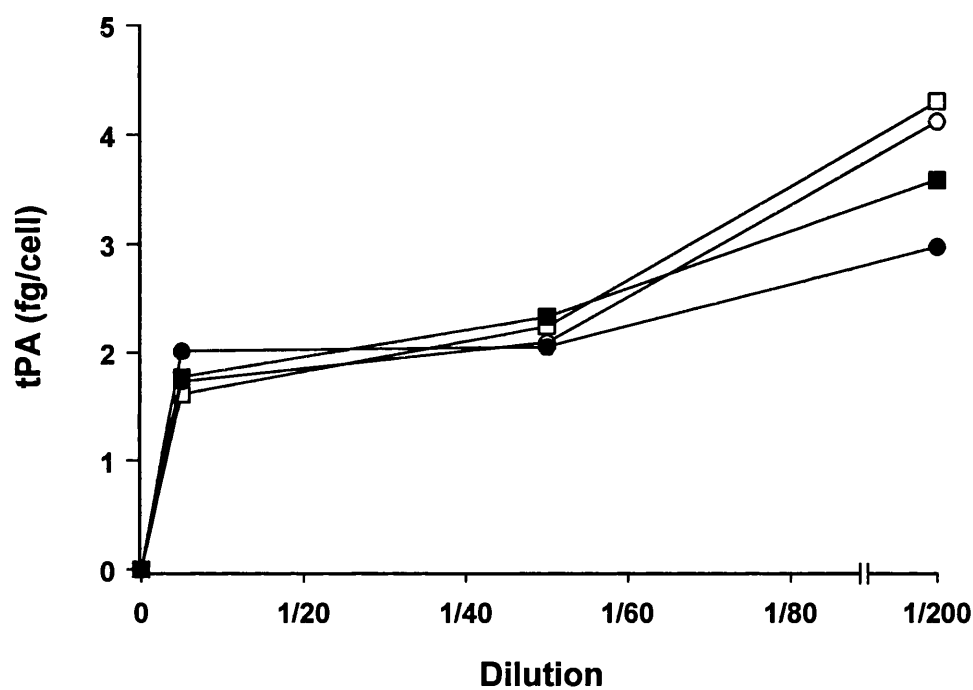


**Figure 43B.** Effect of PX (■10µg/ml; ○100µg/ml; ●250µg/ml; □control) on secretion of uPA from the Hs294T cell line. Figure 35 was used to determine uPA expression and all samples were performed in duplicate with values expressed as fg uPA/cell.

The effect of PX on uPA expression from the A375 cells (which had undetectable levels of secreted uPA) has yet to be analysed.

**3.12. Pentoxifylline modulation of tPA expression**

The effect of PX on tPA secretion from the C8161 cell line was marginal, having a slight effect at the higher dilution where 250µg/ml PX induced a 1.4-fold decrease in tPA expression (Figure 44). As the experiment has only been performed once, it would be necessary to repeat the experiment to confirm the data. The effect of PX on tPA secretion from the Hs294T and A375 cell lines has not been determined.

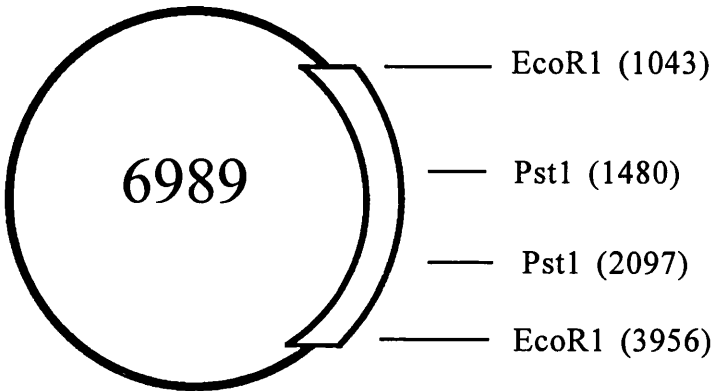


**Figure 44.** The effect of PX (■10ug/ml; ○100ug/ml; ●250ug/ml; □control) on secretion of tPA expression in the C8161 cell line. Data represents the average of duplicate samples.

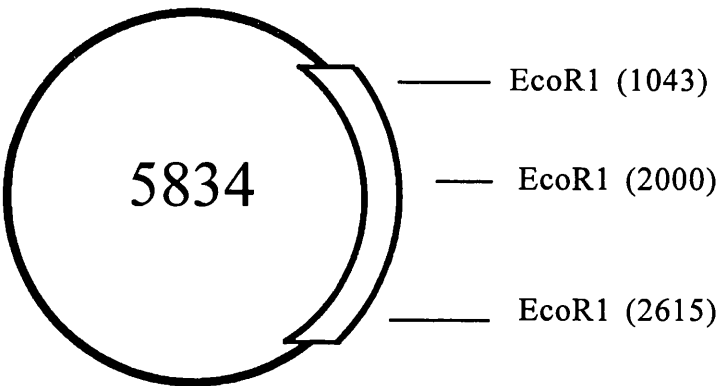
**3.13. Analysis of retinoic acid receptor expression**

The following pSG5 vectors (Figure 45) containing either RAR $\alpha$ ,  $\beta$  or  $\gamma$  inserts provided by P. Chambon (Inserm, France) were transformed into *E. coli*, purified and subjected to restriction enzyme digestion to confirm their identities.

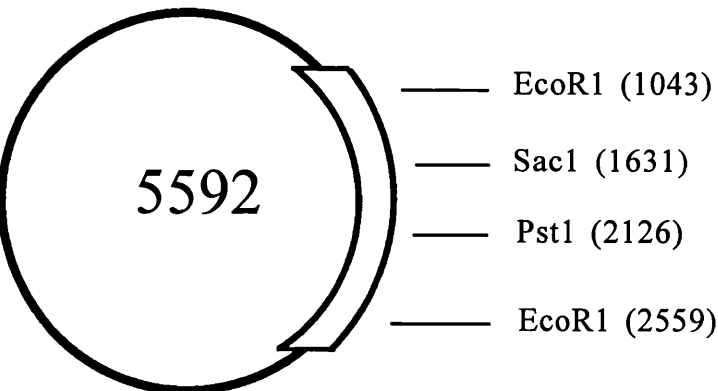
**RAR $\alpha$  vector**



**RAR $\beta$  vector**



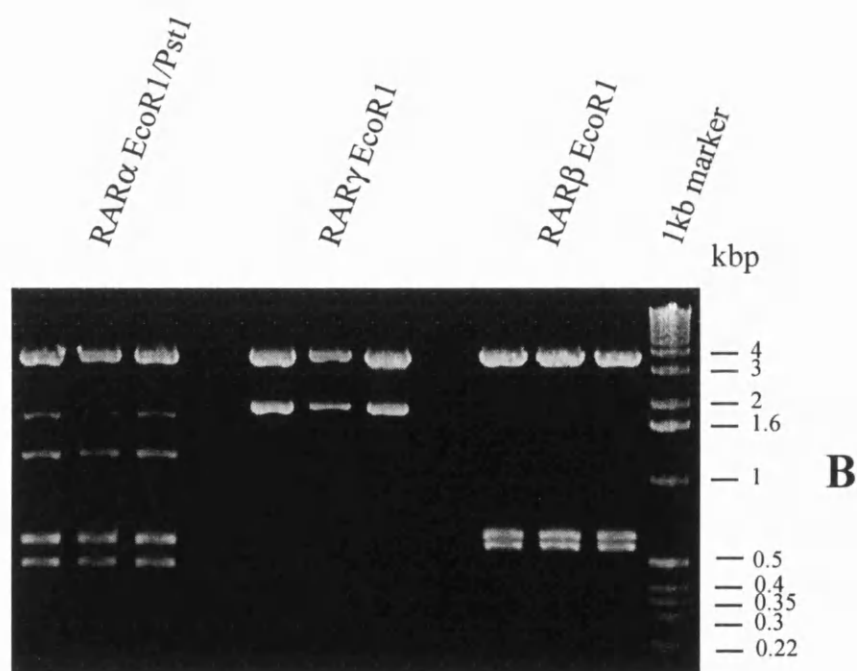
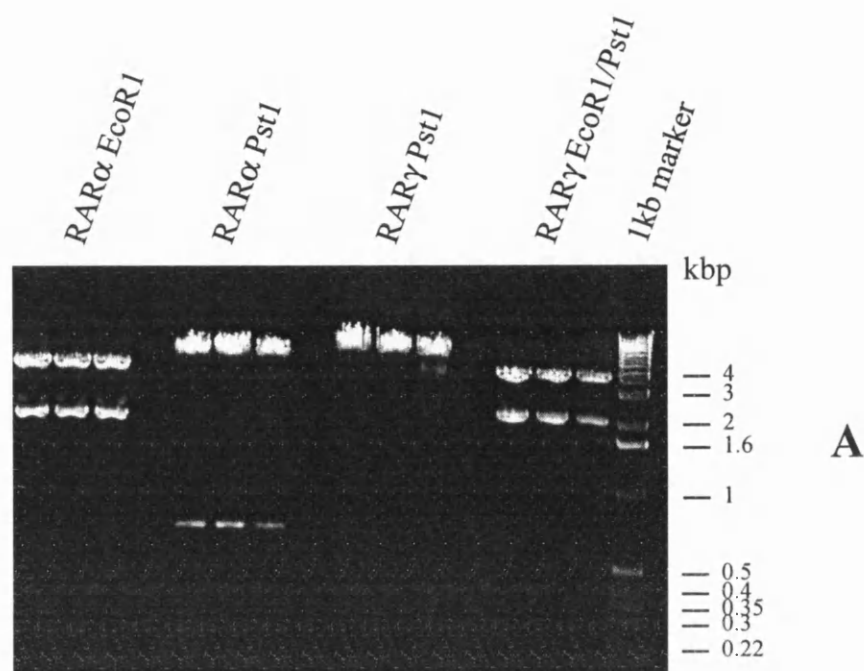
**RAR $\gamma$  vector**

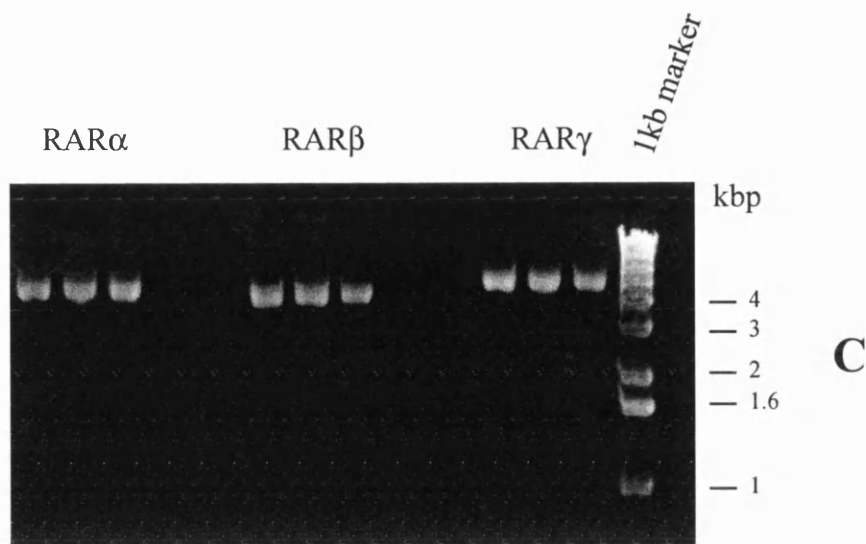


**Figure 45.** pSG5 vectors containing RAR $\alpha$ ,  $\beta$  and  $\gamma$  inserts.

*Restriction enzyme digests of pSG5 RAR $\alpha$ ,  $\beta$  and  $\gamma$*

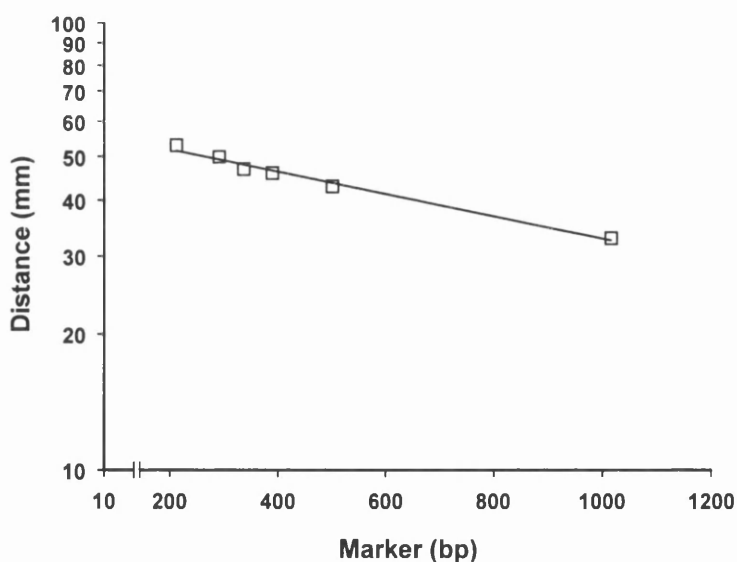
Restriction digests of 3 clones of each pSG5 RAR $\alpha$ ,  $\beta$  and  $\gamma$  gave the following patterns when visualised after agarose gel electrophoresis.



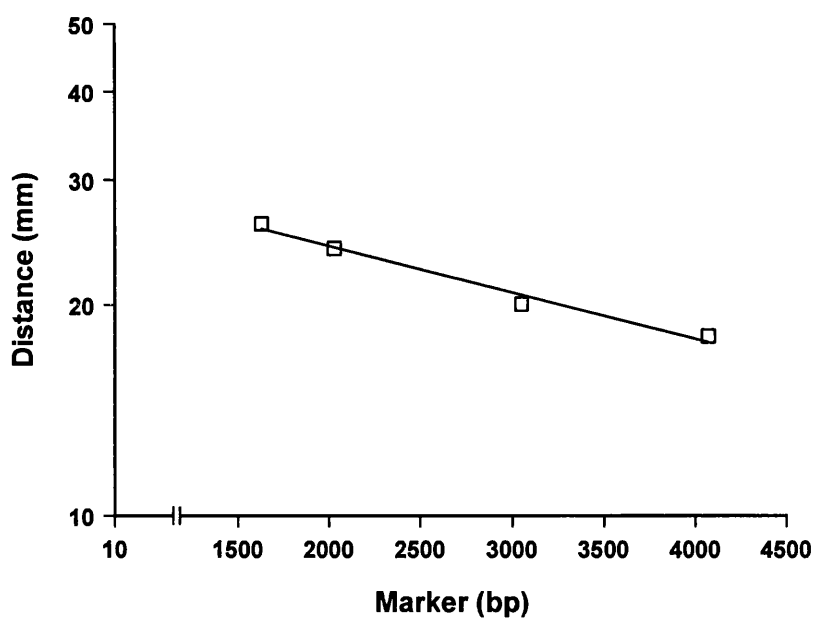


**Figure 46.** Restriction enzyme digestion of RAR $\alpha$ ,  $\beta$  and  $\gamma$  pSG5 vectors

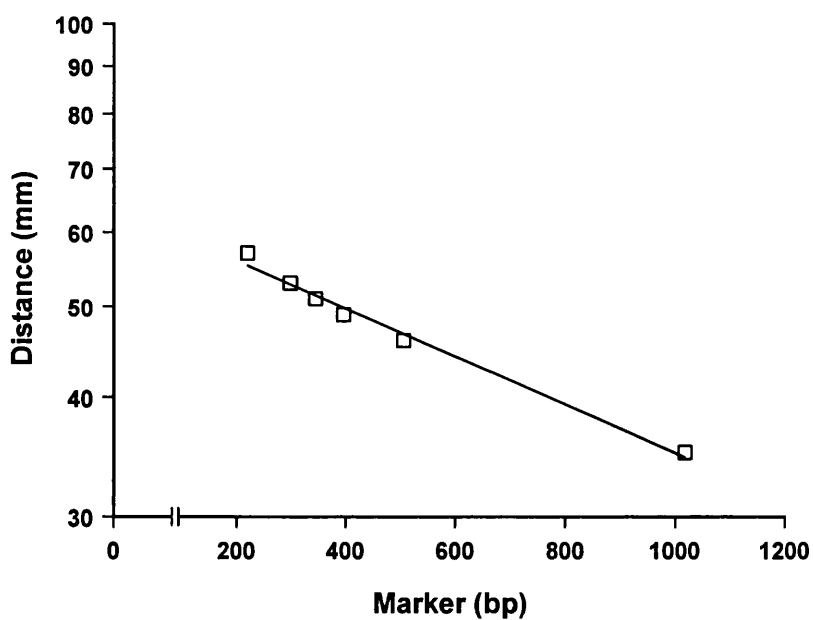
The distance migrated by each band of the marker was measured and a graph plotted of the size in base pairs, versus log distance migrated. This was used to determine the sizes of the bands produced from the digests. Since the marker used was covering a wide range of sizes, 2 separate lines were plotted to produce a linear plot from which the unknown sizes could be determined.



**Figure 47A.** Standard curve of the marker from Figure 46A covering the size range of 220 - 1000bp.

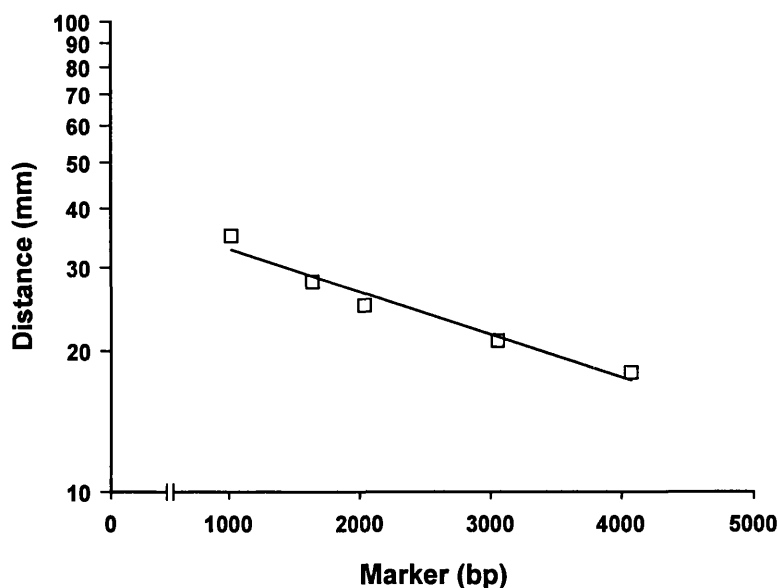


**Figure 47B.** Standard curve of the marker from Figure 46A covering the size range of 1600 - 4000bp.



**Figure 48A.** Standard curve of the marker from Figure 46B covering the size range of 200 - 1000bp.





**Figure 48B.** Standard curve of the marker from Figure 46B covering the size range of 1000 - 4500bp.

The distance migrated by each of the sample bands was measured to allow an approximate size of fragment (in base pairs) to be determined.

Vector	Enzyme Digest	Expected Result (b)	Observed Result (b)
pSG5 alpha	EcoR1	4076, 2913	4000, 1900
	Pst1	6372, 617	>4000, 720
	EcoR1/Pst1	4076, 1859, 617, 437	4000, 1900, (1300), 720, 600
pSG5 beta	EcoR1	4262, 957, 615,	4000, 720, 600
pSG5 gamma	EcoR1	4076, 1516	4000, 2500
	Pst1	5592	>4000
	EcoR1/Pst1	4076, 1083, 433	4000, 2500

**Table 10.** Size determination of the restriction enzyme fragments produced in Figure 46. The value in brackets denotes a possible partial digest product.

The EcoR1 digest product of pSG5 RAR $\alpha$  vector (Table 10, Figure 46A) produced the expected size of band corresponding to cut vector (4kb) and also a second band (1.9kb) corresponding to the insert which was approximately 1kb shorter than the expected size (Table 10). The Pst1 digest product (Table 10, Figure 46A) was similar in size to the expected value (720 bases compared to 617 bases), and the double digest EcoR1/Pst1 of the RAR $\alpha$  vector (Table 10, Figure 46A) confirmed the missing 1kb from the insert. A partial digest product of 1.3kb was also observed and this corresponded to either the digestion product between the EcoR1 site at position 1043 with the Pst1 site at position 2097.

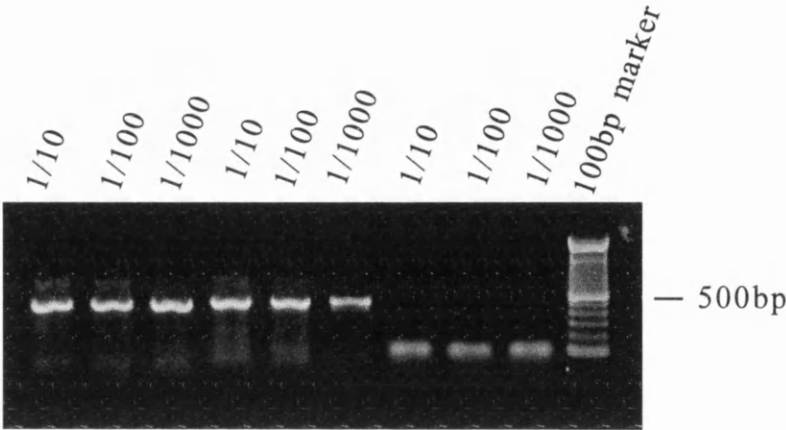
The expected pattern of results was produced for the pSG5 RAR $\beta$  vector with EcoR1 digestion (Table 10, Figure 46B) yielding a fragment of 4kb corresponding to the cut vector and 2 fragments of 0.72kb and 0.6kb corresponding to the insert containing an internal EcoR1 site, although the 0.72kb fragments appeared to be approximately 300 bases smaller than expected.

EcoR1 digestion of the pSG5 RAR $\gamma$  vector (Table 10, Figure 46B) produced the expected size of band of 4kb corresponding to the cut vector whereas the insert appeared to be 1kb larger (2.5kb) than the expected size of 1.5kb. The insert also appeared to lack an internal Pst1 site as there was no corresponding band of 0.4kb visible on the gel (Table 10, Figure 46A) using the double digest of EcoR1/Pst1.

The results produced from these digests raised serious doubts as to the identity of the supplied vectors. Therefore in an attempt to further characterise the probes, partial sequence analysis was performed from amplified regions of each insert.

*Amplification of the pSG5 Vector using Specific Internal Primers.*

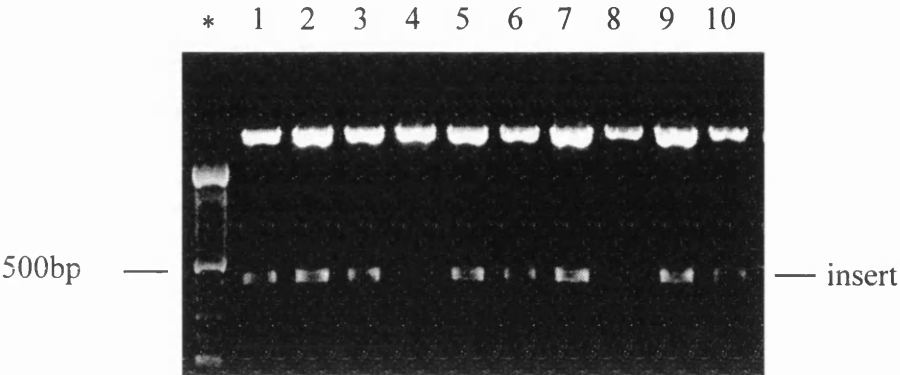
Amplification of the pSG5 RAR $\alpha$  and  $\beta$  vectors was successful for all dilutions producing the expected bands of approximately 500 bases (Figure 49, lanes 1-3 for pSG5 RAR $\alpha$  and lanes 4-6 for pSG5 RAR $\beta$ ). PCR of the pSG5 RAR $\gamma$  vector was unsuccessful using the chosen primers (Figure 49, lanes 7-9).



**Figure 49.** Amplification of pSG5 vectors containing RAR $\alpha$  (lanes 1-3), RAR $\beta$  (lanes 4-6) and RAR $\gamma$  (lanes 7-9). \* denotes the 100bp marker.

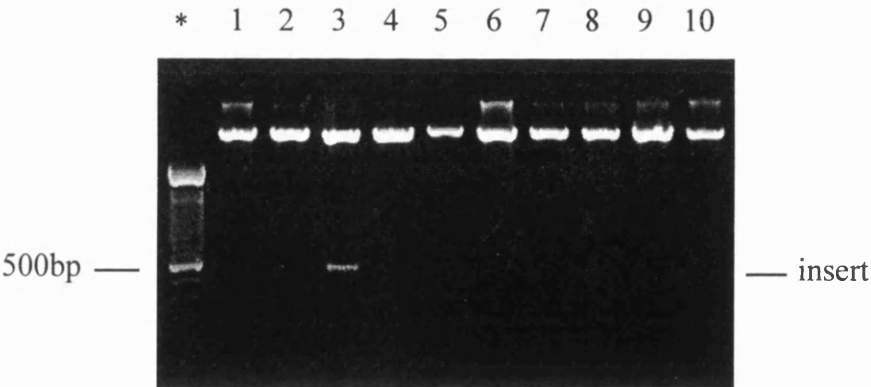
*EcoRI* restriction enzyme digests of the purified pCR2.1 vector from the RAR $\alpha$  and  $\beta$  transformants, for use in the sequencing reactions.

Out of the ten clones picked from the colonies grown after transformation of *E.coli* with pCR2.1 RAR $\alpha$ , 8 out of the 10 contained vector and insert (Figure 50, lanes 1-3, 5-7, 9-10). 2 of the clones contained vector only (lanes 4 and 8).



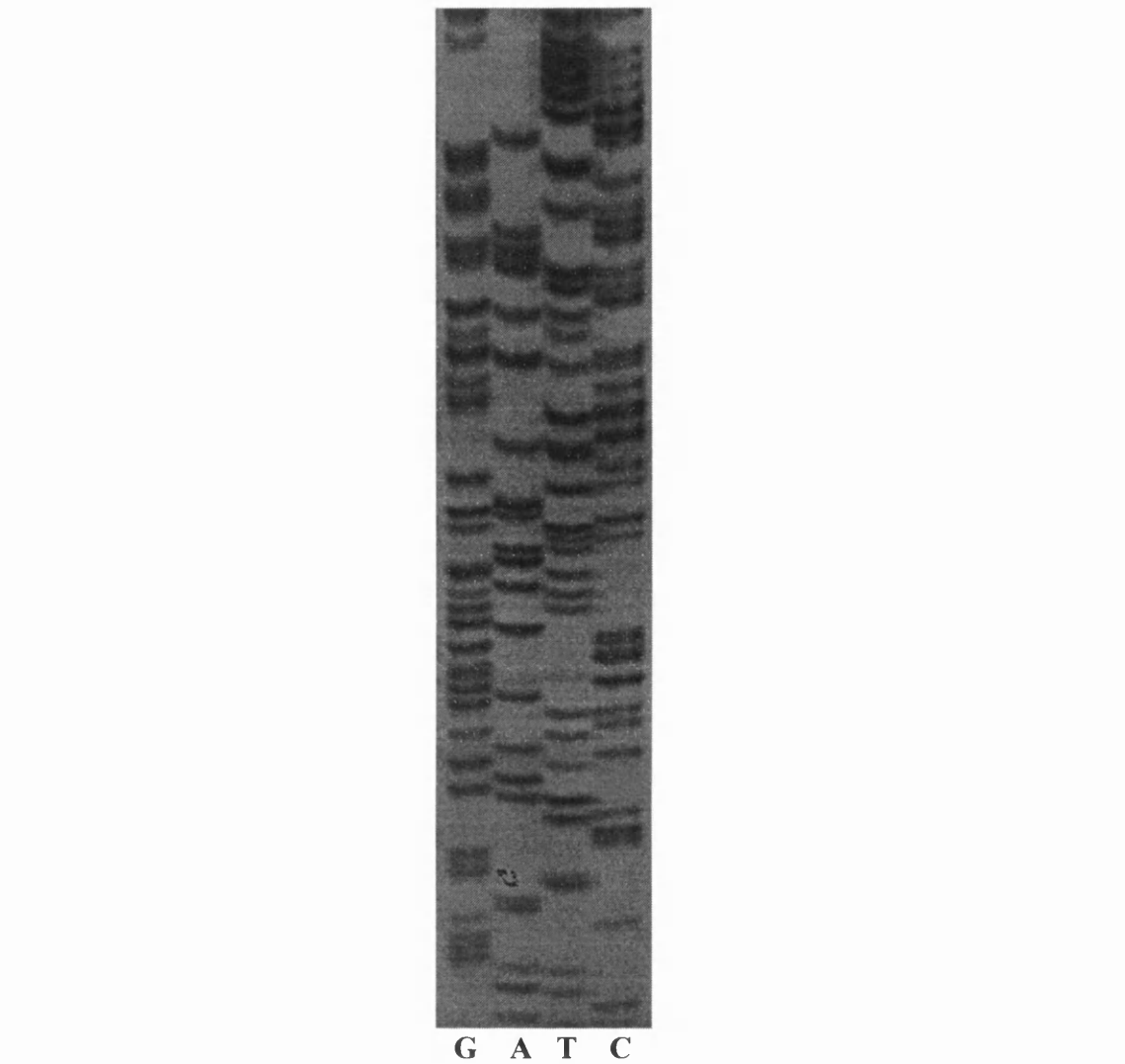
**Figure 50.** *EcoRI* digestion of the RAR $\alpha$  transformation reactions.

Only one clone selected from the pCR2.1 RAR $\beta$  transformations contained vector and insert (Figure 50B, lane 3) whilst the other 9 contained vector only.



**Figure 50B.** *EcoRI* digestion of the RAR $\beta$  transformation reactions.

As larger quantities of vector were required for sequencing, the clones from lane 2, Figure 50A and lane 3 Figure 50B were cultured in 100ml LB broth. The purified vector was sequenced (Figure 51A for RAR $\alpha$  and Figure 51B for RAR $\beta$ ).



E 5'..ACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGAT  
O 5'..ACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGAT

VECTOR

INSERT

←

→

ATCTGCAGAATTCGGCTT**ACCTCCGGCGTCAGCGTGTAGCTCTCAGAGCAC**  
ATCTGCAGAATTCGGCTT**ACCTCCGGCGTCAGCGT** \*TAGCTCTCAGAGCAC

**TCGGGCTTGGGCACCTCCTTCTTCTTCTTGTTTCGGTCGTTT....3' E**  
**TCGGGCTTGGGCACCTCCTTCTTCTTCTTGTTTCGGTCGTTT....3' O**

**Figure 51A.** Partial sequencing of the RAR $\alpha$  PCR product contained within the pCR2.1 vector. \* denotes a missing base. The letters E and O represent "expected" and "observed" sequences respectively.



VECTOR      INSERT

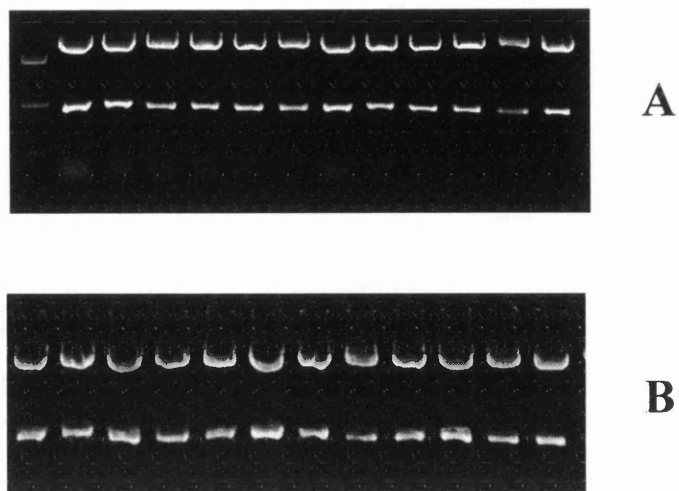
←————→

AGGCCGCCTGCCTGGACATCCTGATTCTTAGAATTT....3' E  
AGGCCGCCTGCCTGGACATCCTGATTCTTAGAATTT....3' O  
↑  
T

162

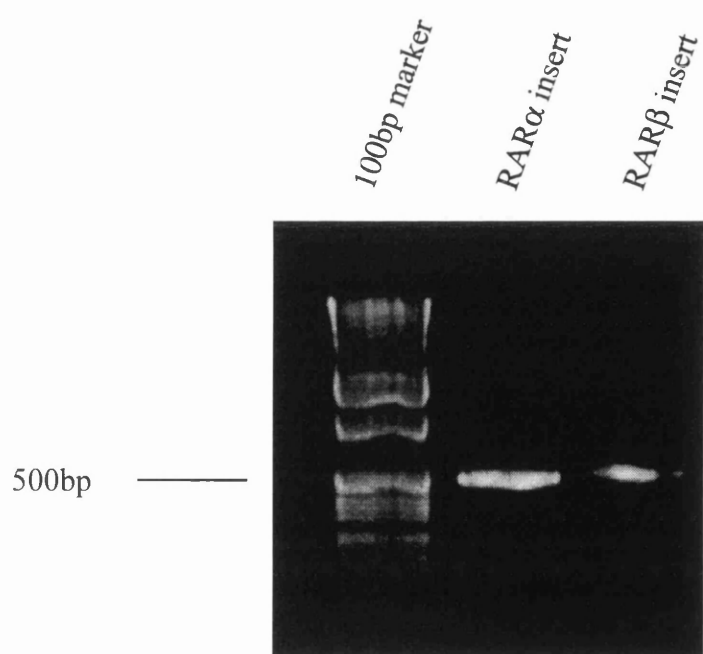
*Preparation of the Probes for Northern Analysis*

The remaining vector was subject to *Eco*R1 digestion (Figure 52) for use as a probe in Northern analysis.



**Figure 52.** Large scale *Eco*R1 digestion of A) pCR2.1 RAR $\alpha$  and B) pCR2.1 RAR $\beta$

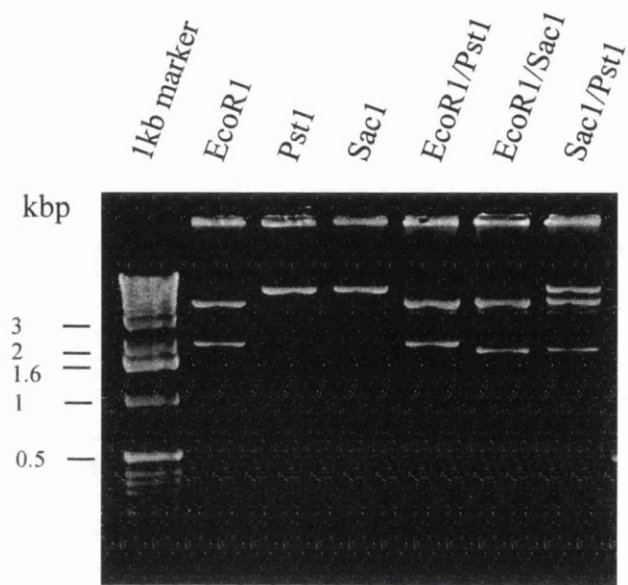
The bands corresponding to the inserts were excised from the gel and purified using the Gel Extraction kit. Successful purification was confirmed by running a small aliquot of each sample on a 1% agarose gel (Figure 53).



**Figure 53.** RAR $\alpha$  and  $\beta$  inserts produced after gel purification

*Restriction Enzyme Digestion of the New Batch of pSG5 RAR $\gamma$*

Restriction enzyme digestion of pSG5 RAR $\gamma$  produced similar results (Figure 54) to the digests on the previous pSG5 RAR $\gamma$  sample (Figure 46) indicating the PstI site was absent from the insert. SacI digestion (Figure 54, lane 4) and the double digests of EcoRI/PstI and EcoRI/SacI (lanes 5 and 6 respectively) and SacI/PstI (lane 7) indicated the lack of a SacI site in the insert.



**Figure 54.** Restriction enzyme digestion of the new pSG5 RAR $\gamma$ .

*Northern Blot Analysis of RAR $\alpha$  and  $\beta$*

Northern blot analysis of RAR $\alpha$  and RAR $\beta$  produced no signal (data not shown) after overnight and 3 weeks exposure of the x-ray film.



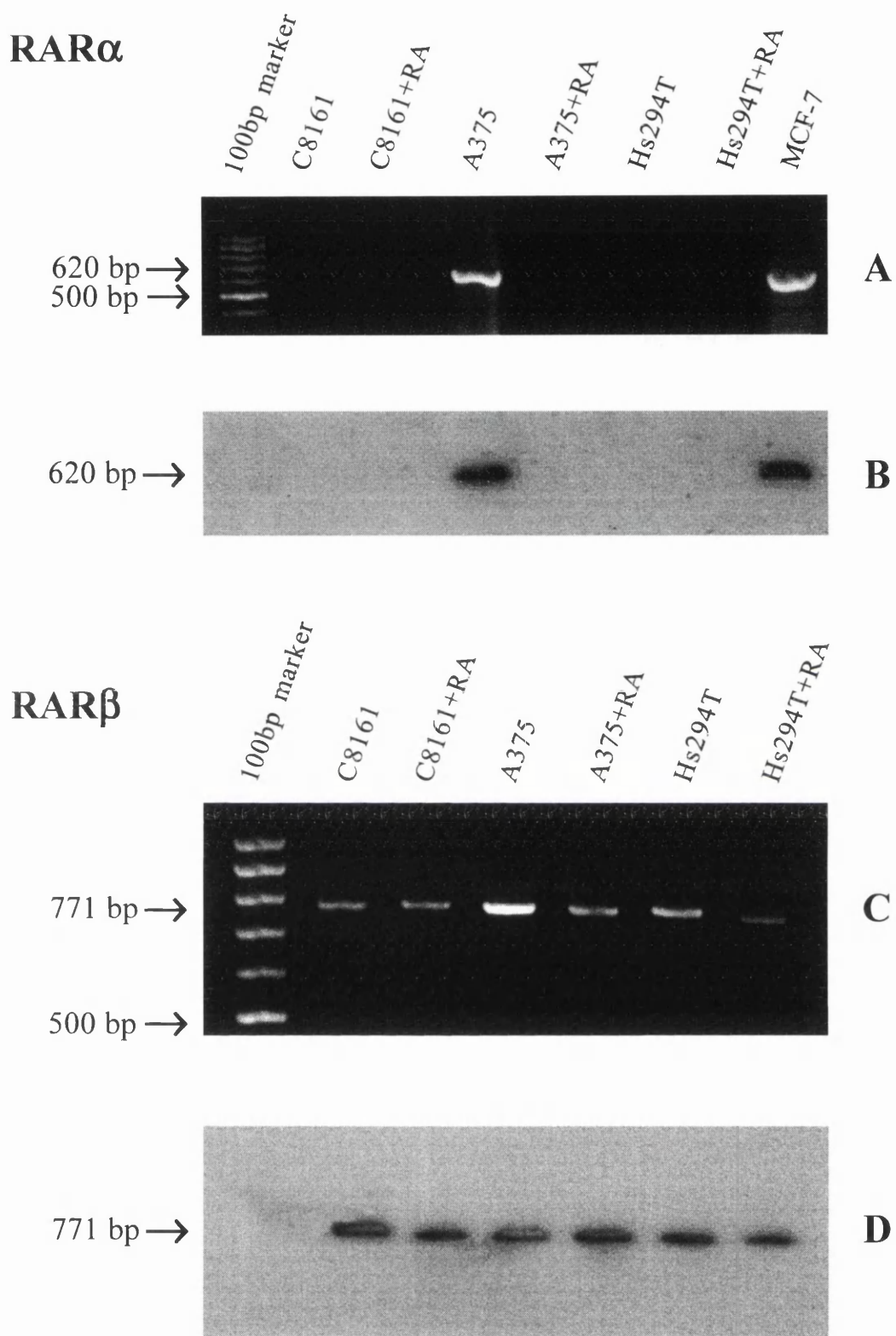
### *PCR amplification and Southern Blotting of RARs*

RAR expression in the cell lines was analysed by RT-PCR due to the failure of the Northern blot approach.

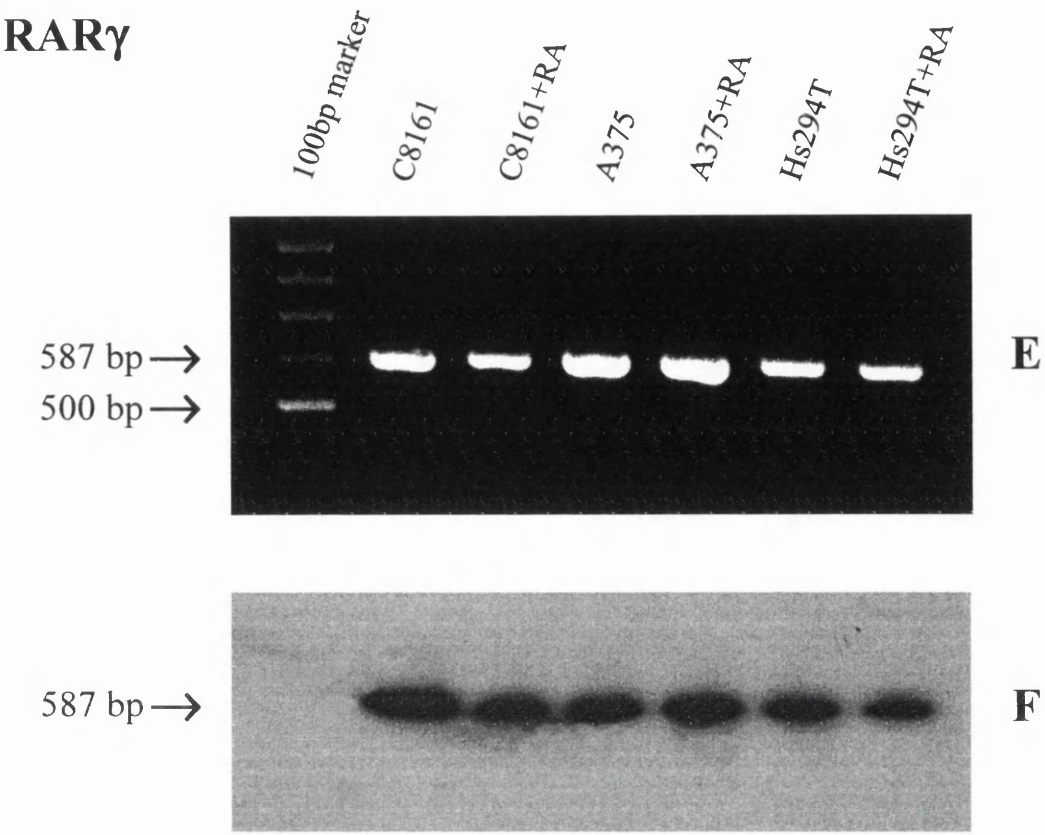
Using RT-PCR, RAR $\alpha$  expression was undetectable in either RA-treated or untreated C8161 and Hs294T cells but was expressed in the control MCF-7 cell line and in untreated A375 cells (Figure 55A). Southern blotting revealed a band corresponding to the MCF-7 and the A375 PCR product (Figure 55B).

Both RAR $\beta$  and  $\gamma$  expression were detectable in treated and untreated cell lines (Figures 55C and 55E respectively) which were confirmed by Southern blotting (Figures 55D and 55F respectively).

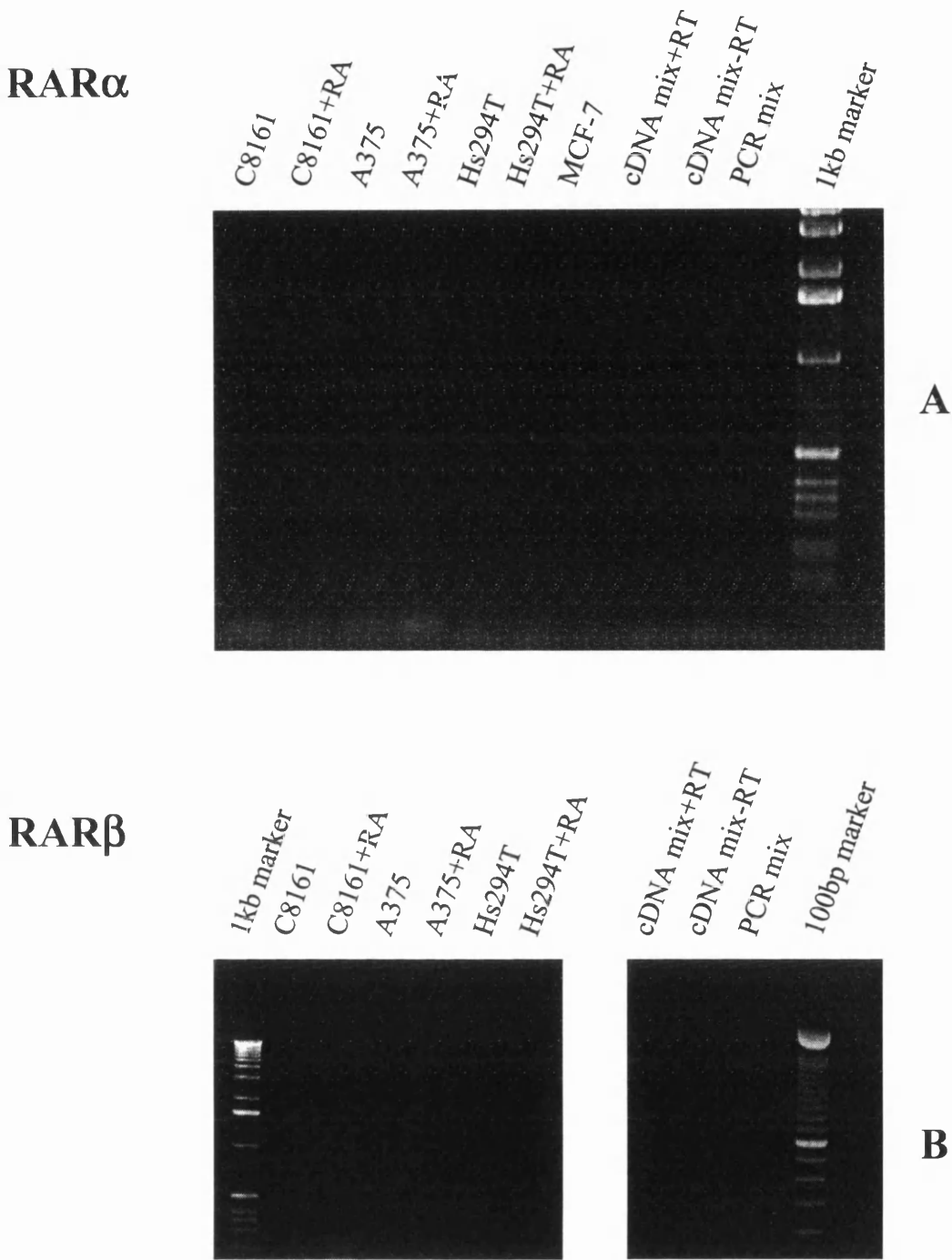
The negative controls of template RNA, cDNA mix minus polymerase, cDNA mix minus RNA template, and PCR mix minus cDNA template were negative for RARs (Figure 56).



**Figure 55A.** Expression of RAR $\alpha$  (A) and RAR $\beta$  (C) in human melanoma cell lines with and without 4 days pretreatment of  $10^{-6}$ M RA. The corresponding Southern blots for RAR $\alpha$  and RAR $\beta$  are shown in Figures B and D respectively.

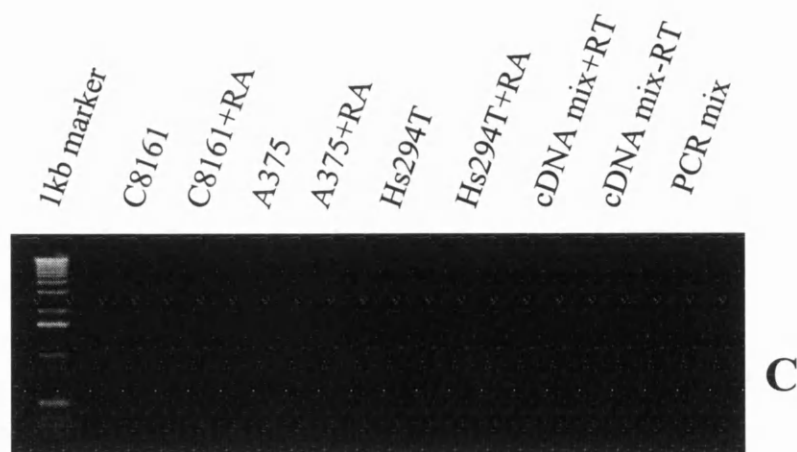


**Figure 55B.** Expression of RAR $\gamma$  (E) in human melanoma cell lines with and without 4 days pretreatment of  $10^{-6}$ M RA. The corresponding Southern blot for RAR $\gamma$  is shown in Figure F.



**Figure 56.** Amplification of RAR $\alpha$  ( A) and RAR $\beta$  (B) using RNA templates from human melanoma cell lines. Other negative controls included were 1) PCR mix minus cDNA template 2) cDNA mix minus reverse transcriptase (RT) 3) cDNA mix with RT.

**RAR $\gamma$**



**Figure 56.** Amplification of RAR $\gamma$  (C) using RNA templates from human melanoma cell lines. Other negative controls included were 1) PCR mix minus template 2) cDNA mix minus RT 3) cDNA mix with RT.

## **Chapter 4. Discussion**

## **Discussion**

### **4.1. Retinoid induced modulation of tumour cell growth and adhesion to ECM components**

The antiproliferative activity of retinoids has been extensively exploited for the treatment of various skin disorders (Kligman et al, 1986) and for the treatment of certain types of cancers (Castaigne et al, 1990; Kraemer et al, 1988; Boone et al, 1990) making the retinoids one of the most prominent chemopreventive agents to reach clinical trials. There have been several reports to show that the retinoids are successful at inhibiting tumour cell proliferation including that from our own laboratory which has shown a decrease in B16 cell growth with RA treatment (Edward et al, 1988). It has been suggested that the RA-induced inhibition in cell growth may involve suppression of c-fos and c-jun which are proto-oncogenes encoding nuclear proteins. They form heterodimers constituting the activator protein complex, AP-1 which is involved in regulating a diverse set of genes important for cell growth and differentiation. Activation of c-fos and c-jun have been shown to induce cellular transformation and Busam et al, (1992) has demonstrated the suppression of c-fos in B16 melanoma cells, suggesting this contributed to the observed decrease in cell growth by RA.

None of the three cell lines tested in this study were responsive to RA-induced growth inhibition (Edward et al, 1997). The exception was using prolonged pretreatment of the tumour cells with RA for 7 days at the higher concentrations which reduced cell numbers. At this point, viability of the tumour cells was also reduced, most likely due to the toxicity of the drug treatment. Hendrix et al, (1990) had previously reported on a 49% decrease in cell numbers using the A375 cells line, however, this data was obtained pretreating the tumour cells with  $10^{-5}$ M RA for 7 days which, in our hands, significantly reduced viability of the tumour cells.

Several recent reports have shown RA has the ability to modulate adhesion of various cell lines to ECM components. Taraboletti et al, (1997) exposed acute promyelocytic leukemia cells to RA and found that adhesion of these cells to subendothelial matrix and to fibronectin was increased. He et al, (1998) demonstrated a RA-induced increase in adherence of NIH3T3 cells to fibronectin, whereas Nakagawa et al, (1998) reported on the ability of RA to increase adhesion of colon adenocarcinoma cell lines to the ECM. Matarrese et al, (1998) also observed an increase in adhesion of RA-treated cervical carcinoma cells to the

ECM.

In our study, analysis of the adherence of C8161 cells to various ECM components was examined, as was the ability of RA to modulate this adherence. Performing the concentration response assays ensured that different concentrations of RA did not affect adhesion since we have shown using the B16 cell line, that RA concentrations did have an effect on B16 cell adhesion to various ECM components (Edward et al, 1989). Since RA was unable to alter adhesion of the C8161 cells to laminin, fibronectin, collagens type I and IV, the effect of RA on adherence of the A375 cells, which had previously been reported to reduce adherence to basement membrane (Hendrix et al, 1990) was investigated. However, the authors used  $10^{-5}$ M RA for 7 days, and in our experiment, pretreatment of the A375 cells for 4 days with  $10^{-6}$ M RA, failed to have a significant effect on tumour cell adherence to basement membrane.

RA has been shown to modulate integrin profile in other cell types such as increasing  $\alpha 3\beta 1$  expression and decreasing  $\alpha 5\beta 1$  expression in embryonal carcinoma cells (Burdal et al, 1994). The  $\beta 4$  integrin subunit was reduced by RA in Lewis lung carcinoma cells which was associated with an inhibition of the malignant phenotype (Gaetano et al, 1994). However, the initial plan to analyse alterations in integrin profile did not merit any further investigation, due to the inability of RA to alter tumour cell adhesion. In any case, very recent studies by He et al, (1998), Nakagawa et al, (1998), and Mataresse et al, (1998) failed to find any changes in integrin molecule profile between control and RA-treated cells, despite showing RA-induced increases in adhesion of the various cell lines to ECM components. Nevertheless, both Nakagawa et al, (1998) and Matarrese et al, (1998) did find an increase in the expression of focal adhesion kinase (FAK) with RA treatment suggesting a potential role for this protein in the RA-induced changes in cell adhesive properties.

#### **4.2. Pentoxifylline-induced modulation of tumour cell growth and adhesion to ECM components**

Tumour cell numbers were decreased in all three cell lines by PX-pretreatment in a dose and time dependent manner which is similar to the recent finding by Amirkhosravi et al (1997), who showed PX significantly decreased proliferation of Neuro2a cells in vitro as well as decreasing the growth of subcutaneously transplanted primary tumours in syngeneic



A/J mice.

PX is known to affect cell adhesive properties, and a report by Franzini et al, (1995) demonstrated the ability of PX to reduce adherence of polymorphonuclear leukocytes to human umbilical vein endothelial cells. Using the C8161 cells, the research in this thesis demonstrates that PX is unable to alter adhesion of the highly metastatic C8161 cells to collagens type I and IV, laminin and basement membrane yet is successful at reducing the adherence of the tumour cells to fibronectin. Our results suggest that PX could possibly alter either expression or activity of those receptors which bind to fibronectin such as  $\alpha 5 \beta 1$  and  $\alpha 3 \beta 1$ , and this could perhaps account for the reduction in adherence to this substrate. Further work will be required to characterise the cell surface receptors involved in binding to fibronectin to explain more fully, the mechanism behind the PX-induced reduction in adherence of the C8161 cells to fibronectin.

#### **4.3. Retinoid-induced modulation of tumour cell lysis**

All three melanoma cell lines in this study were susceptible to attack by LAK cells to a varied extent. This variation depended on the origin of lymphocytes in use, as different donors produced lymphocytes of varying capacity to lyse target tumour cells. It would have been interesting to compare the susceptibility of the low metastatic cell lines with the highly metastatic C8161 cells. However, due to the variability between donors, such an experiment would have required the same lymphocytes to be used in all the assays. This was not practical since a large numbers of lymphocytes were needed for each assay which in turn, would have required large volumes of blood to be taken from one individual. Marrani et al (1994) reported on a microassay version of the classical chromium-51 release assay which used much smaller numbers of tumour cells. Therefore, in producing the E:T ratio, less lymphocytes were required. But, on attempting to reduce the number of tumour cells, the maximum release of chromium-51 from the cells (as measured in dpm) was extremely low. Therefore, the classical chromium-51 release assay was used in this study, hence it was not possible to compare the susceptibility of low with high metastatic cells as different lymphocytes were used for each separate assay. Despite this, the difference induced on drug treatment was always similar despite the variations in lytic capacity of the effector cells. Both RA and PX are known to affect lymphocytes as well as tumour cells, nevertheless, it was hoped in the assays, that by washing the tumour cells at least four

times to remove excess chromium, any residual drug on the tumour cells would be removed and would not interfere with the lymphocytic response.

Recently, Chao et al, (1997) showed that RA decreased the susceptibility of gastric cancer cell lines towards LAK-cell mediated lysis. In contrast, the data produced in our study demonstrated that the C8161 and Hs294T human melanoma cell lines became more susceptible to lysis on treatment with RA. We speculated that alterations in mICAM-1 levels may be responsible for this RA-induced modulation of lysis as alterations in mICAM-1 expression have been correlated with changes of the immune response to tumours. For example, increases in ICAM-1 expression have previously been reported to increase LAK cell-mediated lysis of bladder cancer cells (Jackson et al, 1993; Campbell et al, 1994). Cytokines such as IFN $\gamma$ , TNF $\alpha$  and IL1 (Rothlein et al, 1988) are also able to induce mICAM-1 levels which has been reported to make the tumour cells more susceptible to lysis by cells of the immune system (Webb et al, 1991).

RA is known to increase mICAM-1 expression in several cell types (Bassi et al, 1995; Wang et al, 1992), including melanoma cells (Bouillon et al, 1991), mast cells (Babina et al, 1997), epithelial cells (Gao and MacKenzie, 1996) and cervical cancer cells (Santin et al, 1998), yet in the study by Chao et al, (1997), they were unable to show any effect of RA on mICAM-1 expression. FACs analysis of mICAM-1 in the A375, Hs294T and C8161 cell lines demonstrated a RA-induced increase in mICAM-1 levels in all three cell lines. Subsequent examination of the correlation between ICAM-1 expression and the susceptibility of RA-treated cell lines to lysis by LAK cells followed. The data generated from our blocking ICAM-1 antibody studies indicate mICAM-1 to be partially involved in the tumour/PBL interaction of both treated and untreated Hs294T/C8161 tumour cells, providing further evidence for the involvement of ICAM-1 in the immune response to tumours (Maio et al, 1989; Vanky et al, 1990; Pandolfi et al, 1992). The increase in mICAM-1 expression in response to RA treatment cannot be solely responsible for the increase in LAK cell-mediated lysis of RA-treated tumour cells, as addition of blocking ICAM-1 antibody to the cytotoxicity assays did not reduce lysis to the same level of control experiments. The involvement of ICAM-1 in RA-treated A375 cells was completely abolished which could possibly be via inactivation of expressed ICAM, as levels were in fact increased with RA. It appears that RA modulates the expression of other molecules in addition to ICAM-1 on the tumour cell surface which could ultimately alter the sensitivity of the tumour cells to LAK cell-mediated lysis. Interest in tumour/lymphocyte biology

has also focused upon how lymphocytes recognise the absence of major histocompatibility complex (MHC) class 1 (HLA-ABC) on tumour cells causing the tumour cells to be lysed (reviewed in Mingari et al, 1997). Reports have shown MHC class 1 expression correlates with an inhibitory effect on LAK cell-mediated killing (Kaufman DS, 1995; Litwin et al, 1994). Little data is available on the effects of RA on MHC class 1 expression, but Gross et al (1992) reported on the induction of MHC class 1 by RA in neuroblastoma cell lines whilst De Vries et al (1986) reported on the enhancement of MHC class 1 by RA in two melanoma cell lines. RA has also been shown to increase MHC class 1 expression in cervical cancer cells (Santin et al, 1998). In contrast to this, we speculated that in our cell lines which had become more susceptible to lysis on drug treatment, this increase might have been partially induced by loss of MHC class 1 expression. However, despite our findings that the tumour cells weakly expressed MHC class 1 molecules, we were unable to show that blocking of the HLA-ABC complex with antibody over a range of concentration to untreated or RA-treated tumour cells had any effect on lysis.

Another possible candidate which may be altered by RA and which is present on the tumour surface is LFA-3 (CD58). This molecule interacts with LFA-2 on the lymphocyte surface (reviewed in Springer TA, 1990). Recently, Gwin et al (1996) demonstrated LFA-3 expression correlates most strongly with LAK sensitivity on melanoma cell lines. Modulation by RA is possible; perhaps RA unveils previously masked LFA-3 binding sites or may induce LFA-3 expression or activity to increase the interaction of tumour cells with LAK cells and mediate lysis of a greater number of tumour cells. Future work would involve characterising the alterations in other cell surface molecules which could further explain the RA modulations of tumour cell lysis.

Previous reports indicate mICAM-1 expression in melanoma correlates with an increased risk of metastasis (Kageshita et al, 1993; Johnson et al, 1989). This somewhat conflicting statement may be explained by the existence of soluble ICAM-1 which can interact with LFA-1 on the lymphocyte and block the lymphocyte from interacting with and lysing the tumour cell. Nude mouse models have previously shown melanoma cells to be a source of sICAM-1 (Giavazzi et al, 1992), a finding supported by our ELISA results which show the C8161, Hs294T and A375 cell lines secrete sICAM-1. There is evidence to show that patients with malignant melanoma who have higher levels of sICAM-1 have a reduced survival rate (Harning et al, 1991). It is interesting to note that in our melanoma cell lines, the highly metastatic C8161 cells secrete 5-fold greater levels of sICAM-1 per cell than

the poorly metastatic Hs294T cells which may contribute to the greater ability of the C8161 cells to metastasise in the experimental metastasis model. Although mICAM-1 levels were increased with RA treatment, sICAM-1 levels remained unaltered in the Hs294T and C8161 cell lines. The mechanisms involved in the release of sICAM-1 are unclear, however, it is possible that the soluble form arises either by differential RNA splicing, as is true for P-selectin (Ishiwata et al, 1994), but no corresponding mRNA has been observed. Budnik et al (1996) have provided evidence which points at the the proteolytic cleavage of mICAM-1 to form sICAM-1. Using protease inhibitors, they were able to inhibit the production of sICAM-1 from keratinocytes and showed, using a minimal peptide protection assay, that the production of sICAM-1 was inhibited without having any effect on mICAM-1 expression. It would seem reasonable to speculate from the ELISA results that the immunological consequences of RA treatment in the C8161 and Hs294T cell lines is not via the induction of sICAM-1 and that the mechanisms of regulation of ICAM-1 and sICAM-1 are independent of one another in these cell lines. This has also been observed by Heymann et al (1995) who reported that the induction of mICAM-1 by certain cytokines had no effect on sICAM-1 expression.

sICAM-1 has been shown to bind to LFA-1 on the LAK cell surface which would ultimately prevent the lymphocytes from interacting with mICAM-1, preventing lysis of the tumour cell. The reports on a functional role for sICAM-1 are conflicting. Various groups have reported on its ability to inhibit cell-cell adhesion events such as inhibition of rhinovirus infection (Marlin et al, 1990); T cell/tumour cell interactions (Becker et al, 1993); and lymphocyte/endothelial cell interactions (Rieckmann et al, 1995), and with regards to melanoma cells, Altomonte et al (1993) and Becker et al, (1991, 1993) reported on the inhibition of melanoma cell lysis by sICAM. However, in contrast, several other groups have reported on the failure of sICAM-1 to inhibit (Staunton et al, 1992; Welder et al, 1993) cell-cell interactions. We demonstrate that introduction of this soluble form to the lysis assay of both the Hs294T cells and the C8161 cells does not alter lysis, an observation supported by recent data from Young et al (1997), who found that addition of sICAM to the cytotoxicity assays of bladder cancer cell lines had no effect on lysis. This result is in view of the finding which shows sICAM-1 has a low affinity for LFA-1 (Reilly et al, 1995). Our data indicates sICAM-1 release from these melanoma cells appears to be unimportant in the evasion of these melanoma cells from the immune system in vitro. However, it does not rule out the possibility that in the in vivo situation, sICAM may exist

bound to other proteins or as a multimeric form which may have a greater potential to bind to LFA-1 sites on the lymphocyte which could contribute to tumour cell evasion. sICAM-1 dimers have been shown to bind to solid phase purified LFA-1 with much higher avidity than monomeric forms (Miller et al, 1995). In the natural state, crosslinking of ICAM-1 occurs which can deliver a signal to neutrophils to yield an oxidative burst (Rothlein et al, 1994) and to T cells to activate expression of surface proteins (Poudrier et al, 1994). Therefore crosslinking of sICAM-1 may indeed occur in vivo which could compete more effectively for LFA-1 than monomers.

sICAM-1 addition to RA-treated C8161 cells reduced lysis, suggesting sICAM-1 interference of the tumour/LAK cell interaction plays a significant role in preventing lysis of RA-treated tumour cells. It appears that the combination of sICAM-1 and the RA-induced alterations in membrane molecules is sufficient to partially prevent lysis of the highly metastatic cells.

#### **4.4. Pentoxifylline-induced modulation of tumour cell lysis**

The PX-mediated effects on LAK cell-mediated lysis appeared to be cell type specific as treatment of the C8161 cells with PX made the tumour cells more susceptible to lysis whereas in contrast, the same treatment of the Hs294T cells made the tumour cells less susceptible to lysis.

Previous reports regarding the effect of PX on mICAM-1 expression have shown that expression of ICAM-1 on LPS-stimulated mononuclear cells was reduced with PX (Parhar et al, 1993), a similar finding with monocytes where PX reduced expression of mICAM-1 (Neuner et al, 1997). Reports have indicated one mechanism of downregulation of ICAM-1 by increased cAMP levels is via the inhibition of TNF $\alpha$  release (Taffet et al, 1989; Doherty et al, 1991). Mizutani and Bonavida, (1994) demonstrated that the treatment of OVC-8 ovarian cancer cells with PX reduced TNF- $\alpha$  mRNA expression and both TNF production and ICAM-1 expression are inhibited by PX on mononuclear cells (Mandi et al, 1995). In contrast, a recent report by Zuckerman et al, (1998) showed that increases in cAMP levels was accompanied by an increase in mICAM-1 expression in rat microglial cell cultures. Although all three cell lines were expressing mICAM-1, we were unable to demonstrate any alterations in mICAM-1 expression in response to a range of concentrations of PX.

Addition of blocking ICAM-1 antibody to PX cells reduced lysis but likewise for RA-treated cells, this reduction was similar to the control cells suggesting other molecules must also be affected by PX. Interestingly, the reduction in lysis of PX-pretreated Hs294T cells on addition of ICAM-1 antibody was less than in control cells and required a higher concentration of antibody before a significant reduction became evident.

PX and caffeine have previously been shown to affect MHC class 1 expression (Erusalimsky et al, 1989; Rosenthal et al, 1993) and certainly in murine cells, PX-enhanced MHC class 1 expression has been shown to be partially blocked by an inhibitor of cAMP-dependent protein kinase A (Rosenthal et al, 1993) suggesting the involvement of cAMP in PX-induced MHC class 1 expression. Although we have yet to analyse the effect of PX on MHC class 1 expression, addition of blocking MHC class 1 antibody failed to alter tumour lysis, suggesting it is unimportant in the tumour/LAK cell response.

Whereas with PX which failed to alter mICAM-1 expression, sICAM-1 levels were greatly increased in all the tested cell lines. Similar results with PX treatment have been produced in vivo where plasma concentrations of circulating sICAM-1 have been shown to increase with PX treatment in patients with sepsis (Boldt et al, 1996). If indeed this is the case, then our data may suggest PX has the potential to alter the putative protease(s) involved in cleavage of mICAM-1 to produce the large increase in sICAM-1 expression, whereas RA is unable to effect such proteases. The cytokine profile of these tumour cells and the ability of PX to alter secretion of the cytokines has not been determined. This may also be another mechanism by which PX alters sICAM-1 expression by modulating cytokine expression since PX is known to affect the release of IL-1, IL-2, IL-6, IL-8 and IFN $\gamma$  (Thanhauser et al, 1993; Neuner et al, 1994).

It has yet to be determined whether this increase in sICAM-1 by PX plays an important role in evasion of these tumour cells from cells of the immune system.

The importance of research into ICAM-1 has contributed to the design of therapeutic agents targetting ICAM-1 expression in the clinical setting. Inhibiting leukocyte adhesion by monoclonal antibodies to ICAM-1, LFA-1 or Mac-1 or using soluble fragments of ICAM-1 and antisense oligonucleotides to inhibit ICAM expression has been attempted. Coupling sICAM to Ig produces a dimeric ICAM-1 which has been shown to block in vitro antigen-specific T cell proliferation whilst coupling of ICAM-1 monoclonal antibodies to the ricin A chain was shown to be cytotoxic to human melanoma cell lines.

Recently, a new concept in tumour/lymphocyte biology has been proposed. It has been known for several years that clustering of ICAM-1 occurs in areas of cell-cell contact (Sanders and Vitetta, 1991). Recently, ICAM-2, which was discovered in 1989 (Staunton et al, 1989), has been shown to be important in the tumour/lymphocyte interaction. Helander et al (1996), demonstrated that tumour cells become sensitised to LAK cells when transfected with chromosome 6. Both the resistant and parental cells express similar amounts of ICAM-2. However, in the transfected cells, ICAM-2 was found to be concentrated in the tips of uropods and not evenly distributed in the membrane like the parent cells. The concentration of ICAM-2 was sufficiently high in the transfectants to allow recognition by LFA-1 to occur, causing lysis of the target cell. This redistribution was controlled by a protein found on chromosome 6 called erzin, which is an actin binding membrane cytoskeleton linker protein (Vaehri et al, 1997). Interestingly, the distribution of erzin has recently been shown to be altered during malignant transformation. The outcome of this work suggests that lymphocyte recognition of target cells may require the redistribution of target molecules such as ICAM-1, 2 and LFA-3. This opens up the possibility that PX, and indeed, RA may not simply alter expression of these molecules, but may also modulate the distribution of target molecules on the cells surface which could result in altered cell lysis.

#### **4.5. Lung tumour colonising potential of the C8161, A375 and Hs294T cell lines and the secretion of uPA and tPA.**

This section of the study has provided further evidence that the C8161 cell line is indeed more metastatic in vivo than either the Hs294T or the A375 cell line. The reservation with the experimental metastasis model is that by injecting the melanoma cells directly into a vein, the initial steps involved in metastasis such as local invasion and extravasation are bypassed. Despite this, the model does provide an indication of the metastatic capabilities of the tumour cell and has been widely accepted as a tool to study metastasis. What was surprising with the data obtained from the experimental metastasis work was the large variation in the number of lung tumours formed between each mice within a group. There are several possibilities for this - 1) the injections may not have been accurate such that the target vein was missed or only a small volume of cells were correctly targeted. This appears the most likely explanation and may explain why some of the animals had zero

counts whilst others in the same group had many more counts; 2) the amount of viable cells injected into each mouse may have varied although this was unlikely as viability of each pool of cells was always greater than 95% prior to the experiment; 3) assuming the same numbers of viable tumour cells were injected into each mouse and that the tumour cells were injected into the target vein, then the data shows that certain mice appear to be much more susceptible to forming lung tumours than other mice within the same experimental group. It is probably a combination of both biological and technical variation which has produced the wide range of results within each group. Increasing the sample size and repetition of the experiment would ensure the variation is not due only to technical error. As a result of this, it remains difficult to conclude whether RA or PX has any effect on the cell lines *in vivo* due to the variation in tumour counts.

The next stage of the project involved analysing the expression of PAs from human melanoma cell lines and examining the modulation of the PA components by RA and PX. An early investigation by Fraki et al, (1979) provided initial evidence for an increase in PAs during metastasis as extracts of primary melanomas and melanoma metastases contained significantly higher levels of plasminogen activators than extracts of naevi.

The confocal microscopic analysis suggested that expression of tPA was significantly lower in the C8161 and Hs294T cell lines in comparison to uPA expression, but this statement would only stand true if the specific activity of the uPA and tPA antibodies were similar. Using the ELISA detection system as outlined in the methods section, the study began by analysing the expression of both uPA and tPA in untreated C8161, Hs294T and A375 cells using a range of dilutions. What clearly became evident was that as the samples were made more dilute, the absorbance values became higher until the sample was so dilute that any PA was undetectable. This pattern was consistent throughout the assays and there exists several possible explanations for this. The results suggest that some factor(s) present in the supernatant has the ability to interfere with antibody binding to the tPA or uPA, and when this factor(s) is diluted out, the uPA/tPA is able to bind to the appropriate antibody, hence the absorbance readings increase. Either the factor(s) could be preventing binding of uPA/tPA to the catching antibody or alternatively, reduce binding of the tagging antibody to uPA/tPA. Interference could take the form of altered conformation of the antibodies, or the uPA/tPA, so that binding is reduced, or indeed, the factor(s) may be large enough to block binding of the analyte to the antibodies. The factor(s) may itself bind to either the tagging or the catching antibody to prevent their binding to uPA/tPA.



There is also the possibility that the inhibitory factor(s) may also influence binding of the detecting antibody such that binding of this antibody to the tagging antibody is reduced which would result in a decreased signal. In all cases, the absorbance would be reduced and only by removing the factor would the optical density readings be a true reflection of the quantity of uPA/tPA present in the sample. Diluting the factor out is one way to overcome this problem. Alternatively, if the experiments were to be repeated, another mechanism would be to charcoal strip the serum as serum contains many constituents which could have the potential to interfere with the ELISA, or to culture the cells under serum free conditions. The foreseeable problem with removing the serum is that the tumour cells would not proliferate, or would do so very slowly.

There exists a general consensus whereby more invasive cell lines express higher levels of uPA, and anti-uPA antibody has been shown to reduce both invasion (Mignatti et al, 1986) and matrix degradation of uPA-positive tumour cells (Reiter et al, 1993). In this study, the C8161 cells were found to secrete on average, 215-fold higher levels of uPA than the Hs294T whilst the A375 cells lacked expression of secreted uPA. High expression of cell-associated uPA was observed in the C8161 and Hs294T cells, with weaker expression in the A375 cells. We speculate that the expression of such high levels of uPA from the C8161 cell line may contribute to the greater ability of these cells to metastasise in the experimental metastasis model, as expression of high levels of uPA have previously been correlated with a more metastatic phenotype (Yu et al, 1988). Metastatic subclones of melanoma cell lines have been shown to express higher levels of uPA compared with non metastatic parental melanoma cell lines (Yu et al, 1988), supporting a role for uPA in the metastatic process.

In contrast to uPA levels, many human tumours have been reported to lack the expression of tPA. Human melanoma lesions (De Vries et al, 1995; Delbaldo et al, 1994) have been shown to produce considerable amounts of tPA and high levels of tPA secreted from malignant melanomas has been correlated with the greater potential of these cells to migrate and degrade the ECM (Meissauer et al, 1992). Increases in tPA expression have also been reported to occur during malignant melanoma progression. Using WM164 cells, Herlyn et al, (1990) showed the more metastatic variant, 451-LU, contained 3-fold higher tPA levels and invasion could be inhibited significantly using anti-tPA polyclonal antibodies in an invasion assay. Both the C8161 and Hs294T cells secrete tPA, but interestingly, in contrast to uPA, the poorly metastatic Hs294T and A375 cells secreted higher levels of

tPA. Since uPA plasmin generation has been shown to be a much more efficient process than tPA when comparing uPA-producing MelJuso cells with tPA-producing MeWo cells (Meissauer et al, 1992), the biological significance of higher tPA expression from the poorly metastatic cell lines remains to be established. In the tissues of tumours of diverse origin, tPA levels are often much lower than in benign tissue whereas uPA levels are consistently increased in tumours of diverse origin (De Vries et al, 1996) which may indicate tPA is less important in uPA-producing tumour cells.

#### **4.5.1. Retinoid-induced alterations in uPA and tPA expression**

Reports indicate that there exists a RARE located within the promoter region of the gene for tPA (Bulens et al, 1995) suggesting RA has the potential to modulate expression of this component. Indeed, Kooistra et al, (1995) and Thompson et al, (1991) reported on the induction of tPA by RA in human endothelial cells, whereas Vangiezen et al, (1993) have reported on the induction of tPA by RA in vivo. The synthesis of tPA was also reported to increase on RA treatment in rat endothelial cells (Emeis et al, 1998). In support of this data, we have demonstrated a RA-induced increase in tPA expression in all three cell lines in a dose-dependent manner. Future studies will involve analysing the biological significance of this increase with the speculation that this RA-induced increase in tPA expression may favour an increase in the invasive potential of the tumour cell, as Tiberio et al, (1997) recently reported on the RA-enhanced human neuroblastoma cell invasion of reconstituted basement membrane, which was associated with the induction of tPA expression and a putative tPA receptor, amphoterin. Previous reports on the effect of RA on uPA expression show RA decreases levels of uPA in prostate carcinoma cells (Waghray et al, 1995). This is in contrast to the study by Miwa et al, (1995), who demonstrated a RA-induced increase in receptor bound uPA in fibrosarcoma cells. We have also demonstrated a RA-induced increase in uPA expression in all three cell lines. However, these results are in contrast to other reports showing RA treatment of prostatic carcinoma cells decreased uPA expression (Waghray and Webber, 1995). All-trans RA treatment of prostate carcinoma cells has also been reported to reduce uPA-mediated degradation of fibronectin and laminin and to decrease the ability of the tumour cells to invade matrigel (Webber and Waghray, 1995). The lack of correlation with our results may reflect the possibility that the RA-mediated alterations in uPA expression may be dependent on specific

tumour cell types in vitro. Whether this increase in total uPA expression corresponds to an overall increase in activated uPA has yet to be determined. Kim et al, (1995) demonstrated that despite RA increasing the total activatable uPA expression in human prostate adenocarcinoma cell lines, there was, in contrast, a decrease in uPA activity. This decrease was thought to be due to an increase in PAI-1 production. The expression of PAI-1/2 have not been determined in the C8161 and Hs294T cell lines, however, it is possible that RA may affect levels of these inhibitors as Tienari et al, (1991) have reported on an upregulation of PAI-1 by RA, and the gene for PAI-2 has been shown to contain a RARE (Schuster et al, 1994) allowing for the potential of PAI-2 to be regulated by RA. RA has also been shown to increase the uPAR and to stimulate plasmin generation (Mustjoki et al, 1998) and the availability and numbers of the uPAR may also reflect the potential of the tumour cell to invade. Therefore, the ability of RA to modulate invasion of these tumour cells may not only be determined by uPA and tPA levels, but also by expression of both inhibitors and surface expression of uPAR.

#### **4.5.2. Pentoxifylline-induced modulation of uPA and tPA expression**

Little data is available on the effects of cAMP-inducing agents such as PX to modulate PA expression. However, the reports on the ability of PX to alter experimental metastasis, suggests PX may affect proteinase expression which would contribute to the observed changes in metastasis. There is a cAMP responsive element in the human tPA gene suggesting PX would have the potential to modulate tPA expression. Yet, in this study, it appears PX has very little effect on expression of tPA, certainly in the C8161 cells.

With regards to uPA expression, Miraylopez et al (1991), reported on the induction of uPA mRNA, protein and activity by cAMP-inducing agents. We have shown that in both the C8161 and Hs294T cell lines, PX induces uPA secretion. In a small series, Markus et al (1984), attributed 77% of plasminogen activator activity in melanoma tissue to uPA, and therefore, increases in uPA by PX may contribute to increases in the overall activation of plasmin.

#### **4.6. Retinoic Acid Receptor Expression**

In this section, the expression of the 3 retinoic acid receptors was analysed and we demonstrated expression of RAR $\beta$  and  $\gamma$  in all 3 human melanoma cell lines. We were unable to demonstrate expression of RAR $\alpha$  in the Hs294T or the C8161 cells and although the A375 cell lines expressed RAR $\alpha$ , this was downregulated on treatment of these tumour cells with RA.

The initial plan of study was to use the vectors from Pierre Chambon to act as probes for Northern blot analysis to allow relative quantification of the results. A fasta data search was carried out to determine the presence of any internal recognition sites which could be used to produce a suitable probe for use in the Northern blot analysis of the RARs. RAR $\alpha$  was found to have 2 internal PstI sites within the insert which could have been used whereas RAR $\beta$  had an internal EcoRI recognition site which would have also been appropriate. RAR $\gamma$  had a SacI and a PstI site within the insert which could have been utilised to produce a smaller probe. However, on restriction enzyme digestion of the vectors, there appeared to be some discrepancies comparing the results with the restriction enzyme maps. The RAR $\alpha$  insert lacked 1kb and RAR $\gamma$  insert lacked the SacI and PstI sites. Therefore, in an attempt to try to partially identify the inserts, primers were chosen to amplify specific regions of the vectors to use in subcloning and sequencing of the fragments. Both RAR $\alpha$  and RAR $\beta$  vectors were amplified successfully however, RAR $\gamma$  failed to become amplified under the selected conditions. Partial sequencing of RAR $\alpha$  and  $\beta$  inserts were successful but, RAR $\gamma$  was unable to be sequenced. The only caution with the RAR $\gamma$  result is that a different primer was used in the sequencing reaction since a different vector was being sequenced (pSG5 rather than the pCR2.1 vector) and any faults with the primer may have accounted for the inability to sequence RAR $\gamma$ . This seemed unlikely as successful sequencing of other samples in the laboratory had been achieved in previous research work. Not only did RAR $\gamma$  lack internal recognition sites, but it was also unable to be sequenced, so a fresh batch of RAR $\gamma$  vector was sent from France. Again, the internal recognition sites were missing and sequencing of the pure vector was unsuccessful. It may have been possible to try to cut out a digest fragment of RAR $\gamma$  from the gel and try to subclone this fragment to reveal the partial identity of the insert, however, it was decided to focus the efforts on RAR $\alpha$  and  $\beta$ .

In producing the probes for RAR $\alpha$  and  $\beta$  there were 2 possibilities; one was to simply take the PCR product and label this. The other method would be to purify the bands from the

gel and use this as a probe. Since pure vector was being amplified and not cDNA (where there is the possibility that regions other than the chosen area may be amplified but which are not visible on the gel), it would be reasonable to simply use the PCR product. However, to be certain that the probe used was definitely RAR $\alpha$  or  $\beta$ , the probe DNA was excised from pCR2.1 RAR $\alpha$  and RAR $\beta$ . The initial attempt to produce a probe of high specificity by end-labelling failed to produce any signal on a blot. The likely reason for this was that this form of labelling only produces probes with low levels of activity since only the end of the probes are labelled. Therefore random labelling was chosen as this produces a probe of much higher specific activity as labelling occurs throughout the probe and not just at the 5' phosphate group. The initial blots of RAR $\alpha$  and RAR $\beta$  produced only non-specific signal. There was a possibility to try to overcome the problem of non-specific binding using poly A columns to purify out the mRNA. This also would be beneficial if the message for the receptors was of a very low level. The other method was to try to load more RNA into the wells such that more expressed RAR signal would be present. However, this method would also increase non-specific binding sites at the same time as increasing specific binding sites and would not have helped the initial problem of non-specific binding. Also, loading of the wells with high amounts of RNA was very problematic as the RNA samples became very viscous. Taking into account that even if the blots for RAR $\alpha$  and  $\beta$  were successful, there still remained the problems with the identity of the RAR $\gamma$  vector. On pricing the polyA columns for purifying mRNA, which were shown to be expensive, and having no idea as to whether the receptors were even being expressed, I decided to analyse the RAR expression using RT-PCR to amplify regions of the cDNA using specific primers, then confirm the identity of the PCR amplicons by Southern blotting. This proved to be successful and we were able to demonstrate expression of RAR $\beta$  and RAR $\gamma$  in all three cell lines with and without RA treatment. High levels of RAR $\gamma$  have also been reported to occur in the RA insensitive F9 teratocarcinoma cell line (Wan et al, 1995) whereas in contrast, acinar pancreatic tumour cells which are unresponsive to RA have been shown to lack expression of RAR $\gamma$ . Rearrangement of RAR $\beta$  due to the insertion of the hepatitis B virus has been reported in human hepatocellular carcinoma (Dejean et al, 1986) whilst loss of RAR $\beta$  has also been shown to be an early event in oral carcinogenesis (Lotan R, 1995). Treatment with RA results in clinical improvement with an increase in RAR $\beta$  mRNA. RAR $\beta$  has been shown to be highly inducible for RA (Hoffmann et al, 1990) however, it was not possible to say whether RA increased RAR $\beta$  expression in the cell lines using the

non-quantitative PCR method. The study successfully shows the absence of RAR $\alpha$  in two cell lines with the downregulation of RAR $\alpha$  in the A375 cell line on treatment with RA. Loss of normal RAR $\alpha$  expression is known to be associated with malignant transformation. Acute promyelocytic leukemia (APL) is the translocation of part of chromosome 17 onto the long arm of chromosome 15. This breakpoint on chromosome 17 is located close to the RAR $\alpha$  gene (Borrow et al, 1990; de The et al, 1990) producing abnormal transcripts for RAR $\alpha$  which may block differentiation at the promyelocytic stage. The RA-induced maturation of APL cells into mature granulocytes (Breitman et al, 1990) is associated with a marked reduction in expression of these abnormal transcripts (Zhu et al, 1995). A stably transfected truncated RAR $\alpha$  (lacking the E/F domain) into F9 cells induces a repression of the retinoid response and the cells reveal a RA-resistant phenotype ie they were blocked in differentiation (Espeseth et al, 1989). In P19 cells selected for RA nonresponsiveness, a rearrangement that results in truncation of 70 amino acids from the c-terminal end of RAR $\alpha$  was detected and this truncated RAR $\alpha$  was found to act as a dominant negative regulator (Pratt et al, 1990). We have recently shown that all-trans RA is unable to inhibit growth of the Hs294T and C8161 cell line (Edward et al, 1997) and may be as a result of lacking the expression of RAR $\alpha$ , as the RARs have been implicated in repression of the transcription factor AP-1 (reviewed in Pfahl M, 1993) which has been shown to influence cell growth. Lee et al, (1998) recently reported on the ability of RA to reduce c-fos expression where antagonists of RAR $\alpha$ -suppressed c-fos activity.

#### **4.7. Correlation of RAR profile with ICAM-1 and PA expression**

A specific role for the various subtypes of RAR has been demonstrated in the retinoid-induced modulation of plasminogen activation. It has been suggested that the RA-induced increase in tPA expression requires the involvement of the RAR $\alpha$  (Kooistra et al, 1995). However, in contrast to this, we were unable to demonstrate expression of RAR $\alpha$  in two of the cell lines in which RA-induced tPA increases occurred. Clearly, there must be another mechanism(s) which exists to regulate the RA-induced increase in tPA expression in these cell lines which does not require the expression of RAR $\alpha$ . Lansink et al, (1996) provided evidence for the involvement of RAR $\beta$  in the induction of tPA by RA in human endothelial cells. We were able to show the presence of RAR $\beta$  and  $\gamma$  in both cell lines and postulate a possible role for these receptor subtypes in the RA regulation of both tPA and

uPA. It is also possible that the RXRs might also be involved as these can form heterodimers with the RARs to regulate target gene expression. The use of specific inhibitors for each receptor subtype would provide further evidence for the involvement of each subtype. RAR $\beta$  has been shown to increase along with ICAM-1 on treatment with RA (Bouillon et al; 1991) and Aoudjit et al, (1995) have postulated a potential role for RAR $\beta$  (and RXR $\alpha$ ) in the upregulation of ICAM-1 by RA. This is in accordance with the study by Cilenti et al, (1995) who have demonstrated the ICAM-1 promoter region binds RXR $\alpha$  and RAR $\beta$  with high efficiency. Certainly, all three cell lines express ICAM-1, which was increased with RA treatment, and it would be interesting to quantitate the expression of RAR $\beta$  to see if indeed it does increase in response to RA. The use of antisense ICAM-1 oligonucleotides would prevent an increase in mICAM-1 levels in response to RA, which may in turn, be able to prevent an induction of RAR $\beta$ . Alternatively, specific antagonists of RAR $\beta$  could be used to see whether preventing RAR $\beta$  from interacting with the target response genes, would prevent increases in mICAM-1 expression.

#### **4.8. Future Studies**

The following areas of research relating to this project merit further investigation:

- 1) Characterising the alterations in adhesion molecule profile induced by PX which would possibly allow an understanding of the mechanisms responsible for the reduced adhesion of PX-pretreated C8161 cells to fibronectin.
- 2) Analysis of the modulation and involvement of cell surface molecules implicated in the tumour/LAK cell interaction may further explain the alterations in tumour lysis by RA and PX.
- 3) Repeat the plasminogen activation ELISAs using charcoal stripped serum cultured cells and measure the activity of the PAs to determine whether the increase in tPA and uPA expression by RA corresponds to an increase in "activated" tPA or uPA.
- 4) Examine the expression of PAI-1, PAI-2 and the uPAR by the three cell lines as RA and PX may also influence expression of these proteins, which could ultimately have a significant effect on the invasiveness of the tumour cells.
- 5) Determine the biological significance of uPA and tPA expression using an in vitro invasion assay system, to test if levels of expression of the PA components correlates with invasive capacity. Examining the effects of RA and PX on the tumour cell's invasiveness would also be of interest.
- 6) Analyse the expression and involvement of other protease systems such as the MMPs which are known to be altered by RA and cAMP inducing agents.
- 7) Use specific RAR antagonists to depict a role for each RAR subtype in the RA-induced increases in ICAM-1, uPA and tPA expression.



## **Chapter 5. References**

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## **Chapter 6. Appendix**

## **Presentations and publications arising from this thesis**

### **Publications**

M Edward, JL Godden, CL Alexander, RM MacKie (1997) Effect of retinoic acid on melanoma cell-derived factor stimulation of fibroblast glycosaminoglycan synthesis. *Melanoma Res* 7: 189-196

CL Alexander, M Edward, RM MacKie. Retinoid induced changes in melanoma cell intercellular adhesion molecule-1 expression and susceptibility to LAK cell-mediated lysis (submitted *Br J Cancer*).

CL Alexander, M Edward, RM MacKie. Pentoxifylline-induced modulation of melanoma cell growth, adhesion and LAK cell-mediated lysis (submitted *Melanoma Res*).

### **Poster Presentations**

Presented at British Association for Cancer Research, Annual Scientific Meeting, Edinburgh, March (1996) CL Alexander, M Edward, RM MacKie. Effect of retinoic acid and pentoxifylline on human C8161 melanoma cell adhesive properties. *Br J Cancer* 73: 69.

Presented at British Association for Cancer Research, Annual Scientific Meeting Ireland, July (1998) CL Alexander, M Edward, RM MacKie. Effect of retinoic acid and pentoxifylline on human melanoma cell expression of urokinase type and tissue type plasminogen activators. *Br J Cancer* 78: 102

### **Oral Presentations**

Presented at the Skin Biology Club, Aberdeen, March (1996) CL Alexander, M Edward, RM MacKie. Effect of retinoic acid and pentoxifylline on adhesion of human melanoma cells to extracellular matrix components.

Presented at the European Organisation for the Research and Treatment of Cancer, Glasgow,

July (1996) CL Alexander, M Edward, RM MacKie. Retinoid and pentoxifylline modulation of human melanoma adhesive properties.

Presented at the European Society for Pigment Cell Research International Scientific Meeting, California, November (1996) CL Alexander, M Edward, RM MacKie. Retinoid and pentoxifylline-induced alterations in membrane and soluble ICAM-1 expression and the immunological consequences in vitro.

Presented at the British Association for Cancer Research, 38th Annual Scientific Meeting, Southampton, March (1997) CL Alexander, M Edward, RM MacKie. Comparison of soluble intercellular adhesion molecule-1 (sICAM-1) expression in human melanoma cell lines treated with retinoic acid and pentoxifylline and the role of sICAM-1 in the interaction with lymphokine activated killer cells. *Br J Cancer* 75: 8

Presented at the British Society for Investigative Dermatology Annual Meeting, Bristol, April (1997) CL Alexander, M Edward, RM MacKie. Effect of retinoic acid on intercellular adhesion molecule-1 expression in highly metastatic C8161 human melanoma cells and the immunological consequence in vitro. *Br J Dermatol* 136: 146

Presented at the Department of Tumour Biology, University of Marburg, Germany, January (1998) CL Alexander, M Edward, RM MacKie. Pentoxifylline and retinoid-induced modulation of human melanoma cell metastasis.

Presented at the Institute of Molecular Pathology, Vienna Biocentre, Vienna, March (1998) CL Alexander, M Edward, RM MacKie. Alterations in tumour cell lysis and sICAM-1 expression by the phosphodiesterase inhibitor, pentoxifylline.

Presented at the National Institutes of Health, Washington DC, April (1998) CL Alexander, M Edward, RM MacKie. Retinoid and pentoxifylline modulation of tumour cell adhesion, LAK cell-mediated lysis and plasminogen activator expression.

Presented at the Skin Cancer Group Meeting, Beatson Institute for Cancer Research, Glasgow, May (1998) CL Alexander, M Edward, RM MacKie. Effect of retinoic acid and pentoxifylline on plasminogen activator expression.

